ROLE OF THE PLASMA MEMBRANE CALCIUM ATPASE AS A NEGATIVE REGULATOR OF ANGIOGENESIS

RHIANNON REBECCA BAGGOTT BSc (Hons)

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Abstract

Angiogenesis is the formation of new blood vessels from pre-existing ones. Unregulated angiogenesis is associated with several diseases such as diabetic retinopathy and tumour growth. Many signal transduction pathways have been implicated in the regulation of angiogenesis such as p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), extracellular signal-related kinase 1/2 (Erk1/2) and of particular interest the calcineurin/nuclear factor of activated T-cell (NFAT) pathway. Inhibition of calcineurin activity by the drug cyclopsorin A (CsA) has been shown to inhibit processes required for successful angiogenesis such as *in vitro* cell migration, tube formation and additionally attenuates corneal angiogenesis *in vivo*. CsA is associated with severe side effects and therefore the identification of an endogenous regulator of this pathway would be beneficial. One possibility is the plasma membrane calcium ATPases (PMCAs). These high affinity calcium extrusion pumps have been shown to interact with calcineurin in mammalian cells and cardiomyocytes and down-regulate the calcineurin/NFAT pathway. This is hypothesised to be due to the interaction between the two proteins which maintains calcineurin in a low calcium micro-environment generated by the calcium removal function of the pump. Interestingly, PMCA4 has been shown to interact with calcineurin in endothelial cells.

The aim of our study was to further our understanding of PMCA4s regulation of the calcineurin/NFAT pathway specifically in endothelial cells and establish if PMCA4 has a role in the regulation of angiogenesis. ‘Gain of function’ by adenoviral over-expression of PMCA4 and ‘loss of function’ by either si-RNA

mediated knockdown of PMCA4 or isolation of PMCA4$^{-/-}$ MLEC were used as models. Over-expression of PMCA4 in HUVEC resulted in inhibition of the calcineurin/NFAT pathway with the opposite result occurring in the case of the knockout of PMCA4, identifying PMCA4 as a negative-regulator of the calcineurin/NFAT pathway in endothelial cells. Over-expression of PMCA4 significantly attenuated VEGF-induced protein and mRNA expression of the pro-angiogenic proteins RCAN1.4 and Cox-2, endothelial cell migration and *in vitro* and *in vivo* tube formation with the opposite result occurring in knockdown or knockout studies, confirming PMCA4 as a down-regulator of angiogenesis. Interestingly, over-expression or knockdown of PMCA4 had no effect on VEGF-induced HUVEC proliferation or Erk1/2 phopshorylation proposing PMCA4 may be a potential inhibitor of angiogenesis without compromising cell survival.

Disruption of the interaction between PMCA4 and calcineurin by generation and ectopic expression of an adenovirus encoding the region of PMCA4 that interacts with calcineurin (428-651) (Ad-ID4) resulted in an increase in NFAT activity, RCAN1.4 protein expression and *in vitro* tube formation. These results identify the mechanism of PMCA4s inhibitory effect of the calcineurin/NFAT pathway and consequently angiogenesis is a result of the interaction between the two proteins.

The novel findings of this study establish PMCA4 as a negative-regulator of the calcineurin/NFAT pathway in endothelial cells and angiogenesis. These results are far reaching and highlight a potential role for PMCA4 as a therapeutic target in a variety of diseases that are associated with pathological angiogenesis.

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Abbreviations

Ad- Adenovirus

AKAP79 - A-Kinase-Anchoring Protein 79

AMD – Age-Related Macular Degeneration

Ang – Angiopoietin

APS – Ammonium Persulfate

ATP – Adenosine Triphosphate

ATPase – Adenosine Triphosphatase

BAEC - Bovine Aortic Endothelial Cells

Bcl-2 – B-Cell Lymphoma 2

Bp – Base Pairs

BSA – Bovine Serum Albumin

Cabin 1 - Calcineurin Binding

Cain - Calcineurin Inhibitor

CaM – Calmodulin

CaMBD – Calmodulin Binding Domain

cAMP – Cyclic Adenosine Monophosphate

CASK - Calcium /Calmodulin Dependent Serine Protein Kinase

cDNA – Complementary Deoxyribonucleic Acid

cGMP – Cyclic Guanosine Monophosphate

CHP - Calcineurin Homologous Protein

CLI - Critical Limb Ischemia

Cn – Calcineurin

Cn A - Calcineurin A

Cn B - Calcineurin B

Cn BD – Calcineurin Binding Domain

Cox – Cyclooxygenase

CRAC - Calcium-Release Activated Calcium Channels

CsA – Cyclosporin A

C_t- Cycle Threshold

DAG - Diacylglycerol

DKO - Double Knockout

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleoside triphosphates

DSCR - Down Syndrome Critical Region

DVT -Deep Vein Thrombosis

ECGM – Endothelial Cell Growth Medium

ECM – Extracellular Matrix

E.coli – Escherichia Coli

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal Growth Factor

eNOS – Endothelial Nitric Oxide Synthase

ER - Endoplasmic Reticulum

Erk – Extracellular Signal-Related Kinase

ET-1 - Endothelin-1

Fab – Fragment antigen binding

FBS – Fetal Bovine Serum

Fc- Fragment crystallisable

FDA - Food and Drug Administration

FGF - Fibroblast Growth Factor

FKBP12 - FK506 Binding Protein

FK506 - Tacrolimus

Flk-1 - Fetal liver kinase-1

Flt-1 – Fms-like tyrosine kinase-1

FRET - Fluorescence Resonance Energy Transfer

GOI – Gene of interest

HDMEC - Human Dermal Microvascular Endothelial Cells

HEK293A – Human Embryonic Kidney 293A

HeLa – Henrietta Lacks

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HKG – Housekeeping gene

Hprt1 – Hypoxanthine phosphoribosyltransferase 1

HRP – Horseradish Peroxidase

Hsp27 - Heat Shock Protein 27

HUVEC- Human Umbilical Vein Endothelial Cells

ICAM – Intercellular adhesion molecule

ID4- 3xFlag-PMCA4b(428-651)

IFL - Irinotecan, Fluorouracil and Leucovorin

IFN-γ – Interferon gamma

Ig - Immunoglobulin

IL - Interleukin

IP – Immunoprecipitation

IP_3 – Inositol 1,4,5-trisphosphate

Kb – Kilobase Pairs

kDa – Kilodalton

KDR - Kinase Insert Domain Containing Receptor

LB – Luria Broth

MAGUK - Membrane associated Guanylate kinase

MAP- Mitogen- Activated Protein

MAPK - Mitogen- Activated Protein Kinase

MAPKK - Mitogen- Activated Protein kinase kinase

MAPKKK - Mitogen- Activated Protein kinase kinase kinase

MCIP - Myocyte-Enriched Calcineurin Interacting Protein

MDCK – Madin-Darby Canine Kidney

MEK – MAPK/Erk Kinase

mGluR - Metabotropic Glutamate Receptors

MLEC- Mouse Lung Endothelial Cells

MMPs – Matrix Metalloproteinases

MOI – Multiplicity of Infection

mRNA - Messenger Ribonucleic Acid

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MW – Molecular Weight

NFAT – Nuclear Factor of Activated T-Cells

NFL - Nerve Fibre Layer

NHERF - Na^+/H^+ Exchanger Regulatory Factor

NHR - NFAT Homology Region

NLS - Nuclear Localisation Sequence

nNOS – Neuronal Nitric Oxide Synthase

NO – Nitric Oxide

NOD – Non-Obese Diabetic

NSAID – Non-Steroidal Anti-Inflammatory Drug

NT- Non-Target

OD – Optical Density

OREBP - Osmotic Response Element Binding Protein

PAD - Peripheral Artery Disease

PAGE – Polyacrylamide Gel Electrophoresis

PBS- Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PC12 – Pheochromocytoma

PDE2 – Phosphodiesterase 2

PDGF - Platelet-Derived Growth Factor

PDGFR - Platelet-Derived Growth Factor Receptor

PDR – Proliferative Diabetic Retinopathy

PDZ – (PSD95/Dlg/ZO-1) Post synaptic density protein, Drosophila disc large tumour suppressor and Zonula occludens-1 protein

PE - Phenylephrine

PEMF - Pulsed Electromagnetic Field

pfu – Particle forming units

PF-4 - Platelet Factor-4

PGE_2 - Prostaglandin E_2

PIP_2 - Phosphatidylinositol (4,5)-Bisphosphate

PISP - PMCA Interacting Single PDZ Protein

PI3K - Phosphatidylinositol-3 Kinase

PKA – Protein Kinase A

PKC – Protein Kinase C

PL BD – Phospholipid Binding Domain

PLC-γ - Phospholipase C-γ

PlGF - Placenta Growth Factor

PMA – Phorbol-12 Myristate 13-Acetate

PMCA- Plasma Membrane Calcium ATPase

PP-2B - Protein Phosphatase2B

PSD - Post-synaptic density

PVDF – Polyvinylidene Difluoride

RA - Rheumatoid Arthritis

RASSF1 - Ras-Associated Factor 1

RCAN – Regulator of Calcineurin

RCC - Renal Cell Carcinoma

RGMEC - Rat Gastric Microvascular Endothelial Cells

RHR - Rel Homology Region

RIPA – Radioimmunoprecipitation Assay

RLU – Relative Light Units

RNA- Ribonucleic Acid

ROP - Retinopathy of Prematurity

RPE - Retinal Pigment Epithelium

RT- Reverse Transcription

SAP - Synapse Associated Protein

SDS – Sodium Dodecyl Sulphate

S.E.- Standard Error

sFlt-1 – Soluble Fms-like tyrosine kinase-1

sh-RNA - Short hairpin Ribonucleic Acid

si-RNA- Small Interfering Ribonucleic Acid

SP – Serine/Proline

SRR – Serine Rich Region

svVEGF - Snake Venom Vascular Endothelial Growth factor

TAC – Transverse Aortic Constriction

TAE - Tris-Acetate-EDTA

Tbr-1 – T-box, brain, 1

TBS- Tris Buffered Saline

TBS-T - Tris Buffered Saline 0.05% Tween®20

TE – Tris-EDTA

TEMED – N,N,N',N'-Tetramethylethylenediamine

TGF – Transforming Growth Factor

TIMPs – Tissue Inhibitor of Metalloproteinases

TM- Transmembrane

Tm – Melting Temperature

TonEBP - Tonicity-responsive Enhancer-Binding Protein

TSP-1 - Thrombospondin-1

UBC – Ubiquitin C

UV – Ultraviolet light

VDI – Vascular Density Index

VEGF- Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

WB – Western Blot

WT- Wild-type

YWHAZ – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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1. CHAPTER ONE

Introduction

1.0 Introduction

A plethora of diseases involve unregulated angiogenesis for progression (Liekens *et al*, 2001; Carmeliet, 2005). Inhibition of angiogenesis by down-regulation of the activity of pro-angiogenic molecules, such as vascular endothelial growth factor (VEGF), has been targeted as a treatment in a number of pathologies such as tumour growth (Willet *et al*, 2004; Hurwitz *et al*, 2004) and retinopathies (Gragoudas *et al*, 2004; Rosenfeld *et al*, 2006). Successful angiogenesis requires several events (Liekens *et al*, 2001; Carmeliet, 2005). These are regulated by a variety of cellular signalling pathways that are activated in response to pro-angiogenic proteins, such as VEGF (reviewed in Takahashi and Shibuya, 2005), one of which is the calcineurin/ nuclear factor of activated T cells (NFAT) signal transduction pathway (Hernández *et al*, 2001). Inhibition of calcineurin and consequently this pathway, by the drug cyclosporin A (CsA), results in down-regulation of angiogenesis both *in vitro* and *in vivo* (Hernández *et al*, 2001). However, several adverse effects have been reported with CsA treatment (Matrínez-Martínez and Redondo, 2004). Endogenous regulators of the calcineurin/NFAT pathway have also been identified including plasma membrane calcium ATPases (PMCAs) (Buch *et al*, 2005; Holton *et al*, 2007; Wu *et al*, 2009). These calcium extrusion pumps have been reported to regulate the activity of a variety of interacting partner proteins (reviewed in Di Leva *et al*, 2008). Specifically, PMCA4 down-regulates calcineurin activity in HEK293 cells and cardiomyocytes (Buch *et al*, 2005; Wu *et al*, 2009). However, the role of PMCAs in the regulation of angiogenesis is unknown. An introduction to angiogenesis, the calcineurin/NFAT pathway and PMCAs will be discussed further in this chapter.

1.1 Angiogenesis

Angiogenesis is defined as the formation of blood vessels from pre-existing ones. It occurs both physiologically, during processes such as development and wound healing where it is tightly regulated and pathologically, (Liekens *et al*, 2001) being associated with over 70 diseases (Carmeliet, 2005) such as diabetic retinopathy, rheumatoid arthritis and tumour growth, where this process becomes unregulated (Liekens *et al*, 2001). Alterations in the angiogenic phenotype, for example to pro-angiogenic or anti-angiogenic, are referred to as the ‘angiogenic switch’. This switch is turned on when pro-angiogenic signals such as hypoxia and pro-angiogenic growth factors exceed anti-angiogenic signals and is turned off in the reverse conditions (Bergers and Benjamin, 2003). A multitude of pro- and anti-angiogenic factors have been identified to date (Table 1.1). Among them, vascular endothelial growth factor (VEGF) has emerged as a key regulator of both physiological and pathological angiogenesis (Takahashi and Shibuya, 2005).

Table 1.1: Activators and Inhibitors of Angiogenesis

	Pro-Angiogenic Factors	Anti-Angiogenic Factors	References
Growth Factors	VEGF		Veikkola and Alitalo, 1999, Ferrara *et al*, 2003
	PlGF		Bussolino *et al*, 1996
	PDGF		Heldin and Westermark, 1999
	TGFα, TGFβ		Bussolino *et al*, 1996; Jackson *et al*, 1997; Pepper, 1997; Sato *et al*, 1993
	EGF		Bussolino *et al*, 1996; Sato *et al*, 1993
	FGF-1, FGF-2		Bussolino *et al*, 1996
Protein Fragments		Angiostatin	O'Reilly *et al*, 1994; Cao, 1999
		Endostatin	O'Reilly *et al*, 1997
		Prolactin	Struman *et al*, 1999
Other	Ang-1		Hayes *et al*, 1999; Koblizek *et al*, 1998
	IL-8		Keane and Strieter, 1999
	NO		Jackson *et al*, 1997
	Cox-2		Daniel *et al*, 1999; Hernández *et al*, 2001
		IL-12, IL-4	Sgadari *et al*, 1996; Volpert *et al*, 1998
		TIMPs	Gomez *et al*, 1997
		TSP-1	Iruela-Arispe and Dvorak, 1997
		PF-4	Moore *et al*, 1998
		Ang-2	Maisonpierre *et al*, 1997
		IFN-γ	Sato *et al*, 1990

1.1.1 Physiological Angiogenesis

1.1.1a Embryonic Development

Successful embryonic development requires the formation of the vasculature for the delivery of oxygen and nutrients required for growth (Patel-Hett and D'Amore, 2011). Vascularisation of the embryo occurs initially via a process termed vasculogenesis which generates the primary vascular network. Angiogenesis then modifies this initial network and extends from it by either intussusceptive or sprouting angiogenesis. Intussusception divides a pre-existing vessel lumen into two by insertion of tissue. In comparison, sprouting involves endothelial cell migration, tube formation and proliferation and will be the focus of our study (Patan, 2000). In the embryo, angiogenesis is initiated by E9.5 days (Patel-Hett and D'Amore, 2011). Inhibition of angiogenesis *in vivo* prevents embryo growth due to attenuation of vascular formation and yolk sac development (Klauber *et al*, 1997). Additionally, the pro-angiogenic factor VEGF has been identified as an essential signalling molecule in embryonic development as knockout of this protein leads to embryonic lethality (Carmeliet *et al*, 1996; Ferrara *et al*, 1996).

1.1.1b Wound Healing

Wound healing is comprised of four sections; haemostasis, inflammation, proliferation and matrix formation and remodelling. Angiogenesis occurs in the proliferation phase (Greaves *et al*, 2013) which is between days 3 and 20 after wound occurrence (Bauer *et al*, 2005) and is essential for the delivery of oxygen and nutrients to the wounded area, the restoration of capillaries that have been injured (Greaves *et al*, 2013) and the removal of debris from the site of injury (Nissen *et al*, 1998). Keratinocytes, platelets, macrophages, neutrophiles,

endothelial cells, fibroblasts and smooth muscle cells are all a source of VEGF in wound sites (Greaves *et al*, 2013) with the most significant signal of VEGF being between 3 and 7 days after initial wound occurrence (Nissen *et al*, 1998). Endothelial cells migrate into the wound site via tips of capillaries towards the VEGF signal which is produced due to the hypoxic conditions found in a wound (Greaves *et al*, 2013).

1.1.1c Menstrual Cycle

The menstrual cycle is divided into three main stages; the menstruation phase, the proliferative phase and the secretory phase. Angiogenesis occurs in all three stages for the restoration of the remaining layer of the endometrium, the development and thickening of the endometrium and the growth and coiling of spiral arterioles to supply the functional layer of the endometrium respectively. (Demir *et al*, 2010; Maas *et al*, 2001; Shifren *et al*, 1996). Interestingly, the angiogenic potential, as measured by the vascular density index (VDI) was shown to be increased in human endometrial fragments from early proliferative and early and late secretory phases, suggesting a requirement for angiogenesis at these specific time points in the menstrual cycle which correspond to periods of vessel growth (Maas *et al*, 2001). Furthermore, VEGF mRNA expression in human endometrium increased throughout the mid and late proliferative and secretory phases compared to the early proliferative phase, with the greatest expression detected in the secretory phase which is associated with the highest angiogenic activity of the endometrium (Shifren *et al*, 1996).

1.1.2 Pathological Angiogenesis

Pathological angiogenesis is the result of unregulated angiogenesis which is associated with a variety of diseases (Liekens *et al*, 2001). This can occur due to either insufficient or excessive angiogenesis (Carmeliet, 2005).

1.1.2a Insufficient Angiogenesis

1.1.2a(i) Limb Ischemia

Critical limb ischemia (CLI) is associated with peripheral artery disease (PAD) (Annex, 2013). It is the result of occlusion of arteries in the lower limbs, for example due to atherosclerosis, that impairs blood flow to the lower extremities consequently causing limb pain, ulcers, gangrene and in severe cases amputation (Annex, 2013). Therapeutic angiogenesis has been shown to alleviate the adverse effects of CLI as seen by an increase in collateral blood vessel formation and ulcer healing in CLI patients treated with intramuscular injection of plasmid DNA encoding VEGF-A_{165} (Baumgartner *et al*, 1998).

Diabetes mellitus has been reported to be a risk factor of CLI, (Annex, 2013). In a non-obese diabetic (NOD) limb ischemia mouse model, that is representative of type I (insulin dependent) diabetes, angiogenesis was shown to be reduced as a decrease in blood flow and capillary density were observed (Rivard *et al*, 1999). Furthermore, wound healing is attenuated in diabetic mice compared to healthy mice, which was suggested to be due to a reduction in VEGF expression which was shown to be down-regulated in the phase of wound healing that requires angiogenesis (Frank *et al*, 1995).

1.1.2b Excessive Angiogenesis

1.1.2b(i) Rheumatoid Arthritis

Rheumatoid arthritis (RA) is associated with two stages; a highly inflammatory phase and a vascular phase (Marrelli *et al*, 2011) with the severity of the disease positively correlated with increasing levels of VEGF (Sone *et al*, 2001b). In RA there is hyperplasia of the synovium resulting in an increase in mass (Maruotti *et al*, 2006) generating a hypoxic environment that leads to an increase in VEGF expression and a concomitant up-regulation of angiogenesis (Paleolog, 2002). Synovial fluid and inflammation is also increased and consequently leads to joint pain and swelling (Maruotti *et al*, 2006). A highly vascularised pannus is formed that damages cartilage and bone and leads to joint destruction (Maruotti *et al*, 2006; Paleolog, 2002). Angiogenesis not only transports oxygen and nutrients required for the proliferation of synovial cells, but also localises inflammatory cells leading to inflammation of the synovium (Marrelli *et al*, 2011).

1.1.2b(ii) Psoriasis

Psoriasis is a chronic inflammatory disorder of the skin resulting in a hyperplastic epidermis, increased angiogenesis, generation of an immune response and localisation of inflammatory cells such as macrophages to this site (Singh *et al*, 2013). In particular microvessels of the papillary dermis have increased permeability, are elongated and dilated. VEGF and VEGF receptors VEGFR-1 and 2 were shown to be over-expressed in biopsies of psoriatic skin compared to control patients especially in the hyperplastic epidermis. It is proposed angiogenesis is augmented in this condition to cope with the

increased demand of oxygen and nutrients at hyperplastic sites (Detmar *et al*, 1994).

1.1.2b(iii) Retinopathies

Angiogenesis has been implicated in the progression of a number of retinopathies. An increase in VEGF levels in the ocular fluid has been reported in retinopathies with neovascularisation. Furthermore, treatments that reduce neovascularisation such as photocoagulation also reduce levels of VEGF (Aiello *et al*, 1994).

Neovascular (wet) age-related macular degeneration (AMD) is most prevalent in the elderly and causes irreversible sight loss (Zhou and Wang, 2006). Choroidal neovascularisation in wet AMD is the result of abnormal blood vessel growth into the retina (Kourlas and Abrams, 2007; Zhou and Wang, 2006) that results in leaking and haemorrhaging of the retina and potentially retinal pigment epithelium (RPE) detachment (Kourlas and Abrams, 2007). Specifically in rats, increases in choroidal blood vessel growth, vascular leakage and retina degeneration were observed in the presence of ectopic expression of VEGF in the RPE (Spilsbury *et al*, 2000).

Diabetic retinopathy is a severe complication associated with diabetes. Proliferative diabetic retinopathy (PDR) displays as macular ischemia and oedema and neovascularisation of the retina resulting in haemorrhage, glaucoma and in severe cases retinal detachment and vision loss (Cheung *et al*, 2010). VEGF expression is increased in retinal endothelial cells and pericytes due to hypoxic conditions within the eye, resulting in an increase in angiogenesis and enhancement of the permeability of capillaries (Cheung *et al*,

2010). Specifically, in patients with PDR, levels of VEGF in the vitreous have been reported to be increased in comparison to non-PDR patients (Adamis *et al*, 1994).

1.1.2b(iv) Endometriosis

Endometriosis is the presence and growth of the endometrium at locations outside of the uterus such as the ovaries and abdominal cavity. It occurs when the lining of the uterus (endometrium) sheds during menstruation (Hull *et al*, 2003). Angiogenesis at these ectopic sites leads to the development of a vascular supply and growth of these lesions which results in a pathological phenotype (Hull *et al*, 2003). Levels of VEGF expression in the peritoneal fluid of women correlate with the severity of the disease (Shifren *et al*, 1996).

1.1.2b(v) Tumour Angiogenesis

When a tumour reaches 1-2mm it requires an enhanced supply of nutrients (Bergers and Benjamin, 2003). Angiogenesis generates new vessels to supply the tumour with oxygen and nutrients to maintain its survival (Papetti and Herman, 2002) and is involved in the metastatic spread of tumours (Jain, 2005). Pathological tumour angiogenesis differs from physiological angiogenesis as the blood vessels formed are abnormal. Tumour vessels are leaky, disorganised (Jain, 2005), dilated and often have dead ends resulting in a reduced blood flow (Bergers and Benjamin, 2003). Pericyte attachment is significantly reduced resulting in disruption of vessel diameter (Bergers and Benjamin, 2003). Growth and proliferation of the tumours results in additional pressure onto the vasculature which constricts them, consequently inhibiting blood flow into tumours and increasing the hypoxic environment (Jain, 2005).

Furthermore, an increase in VEGF expression is associated with augmentation of microvessel density and a decrease in the relapse free survival rate of patients compared to tumours with less VEGF (Toi *et al*, 1994).

1.1.3 Processes Required for Successful Angiogenesis

Angiogenesis is a highly complex process (Liekens *et al*, 2001) consisting of several events such as basement membrane break down, migration and tube formation (Fig. 1.1.1) (Carmeliet, 2000).

1.1.3a Basement Membrane Degradation

In response to pro-angiogenic factors, pericytes or smooth muscle cells are removed from the existing vessel (Papetti and Herman, 2002) which results in their destabilisation (Carmeliet, 2000), allowing endothelial cells to respond to environmental factors (Liekens *et al*, 2001). In an environment favouring angiogenesis initiation, endothelial cells release proteases such as plasmin and matrix metalloproteinases (MMPs) in response to pro-angiogenic signals to degrade the basement membrane. (Liekens *et al*, 2001).

1.1.3b Cell Migration and Proliferation

The degradation of the basement membrane allows the migration of endothelial cells out of the existing blood vessel into the surrounding tissue (Fig 1.1.1) and releases pro-angiogenic factors associated to the extracellular matrix (ECM) which can activate the proliferation of endothelial cells (Liekens *et al*, 2001; Conway *et al*, 2001). The loss of cell-cell and cell-ECM interactions is also required for the successful migration of endothelial cells across the opened

basement membrane. Proliferating cells follow the migrating cells to form solid tubes (Liekens *et al*, 2001).

1.1.3c Tube Formation

Functional tubes are generated by the formation of a lumen in the migrated endothelial cells that have formed tubular like structures. This process occurs by the thinning of endothelial cells and involves a variety of growth factors and proteins (Conway *et al*, 2001). Even though new blood vessels have been generated they are still termed ‘immature’ as they require stabilisation via interaction with the ECM (Liekens *et al*, 2001) or the recruitment of pericyte or smooth muscle cells to the vessel wall (Carmeliet, 2000).

1.1.3d Recruitment of Pericyte and Smooth Muscle Cells

The final stage of angiogenesis is the recruitment of pericytes or smooth muscle cells to the newly formed blood vessel to construct a mature, functional vessel (Fig 1.1.1) (Carmeliet, 2000). These peri-endothelial cells regulate the vessel diameter and increase the contacts between endothelial cells. They play a part in the formation of the basement membrane and generate proteins of the ECM (Bauer *et al*, 2005). Additionally, they also act to prevent any further cell proliferation, migration or vascular permeability, regulate blood flow and inhibit the regression of the vessel acting as a stabilisation mechanism (Conway *et al*, 2001).

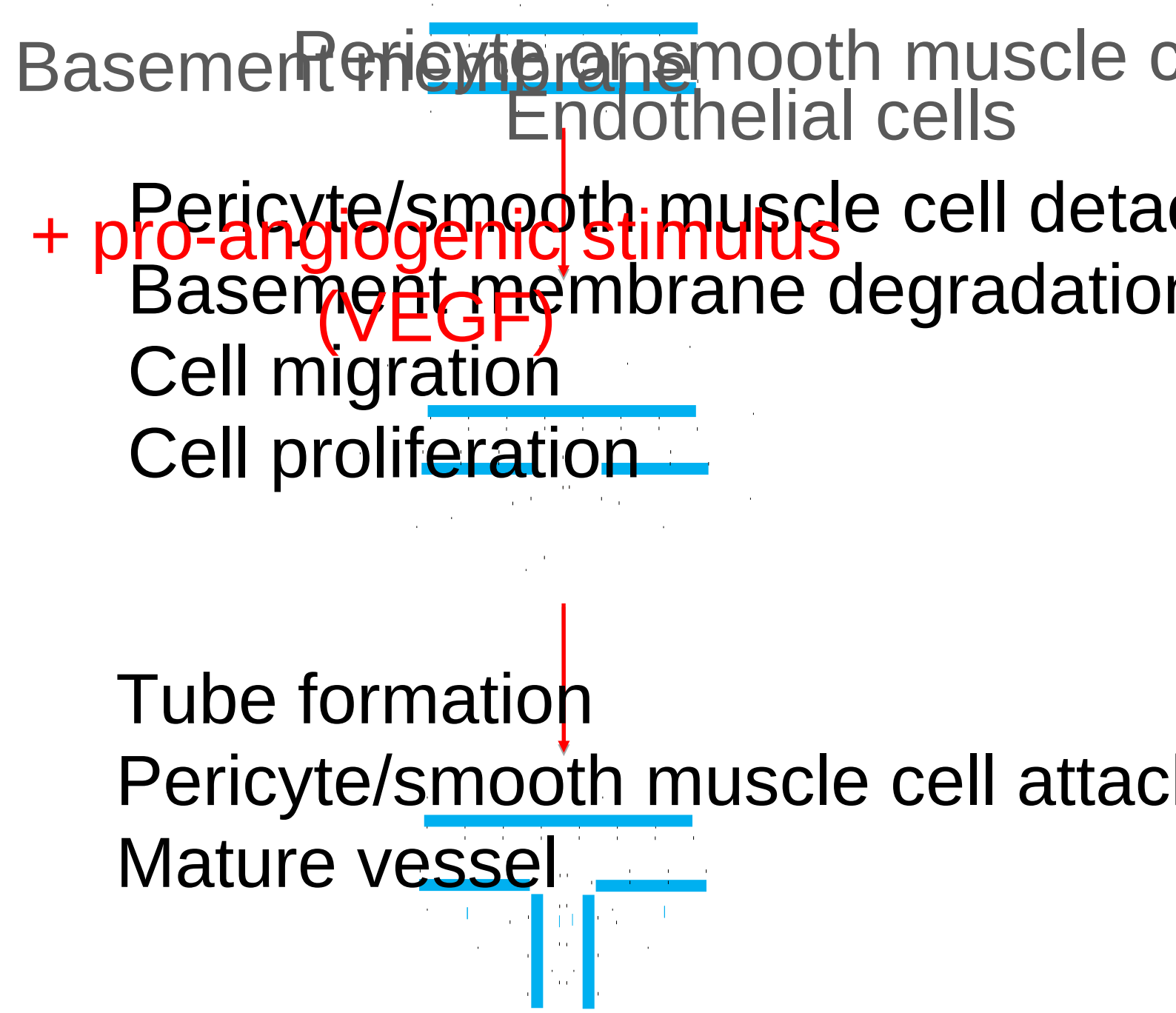

Basement membrane
Pericyte or smooth muscle c
Endothelial cells
Pericyte/smooth muscle cell detac
+ pro-angiogenic stimulus
(VEGF)
Basement membrane degradation
Cell migration
Cell proliferation
Tube formation
Pericyte/smooth muscle cell attac
Mature vessel

Fig. 1.1.1 Mechanisms required for successful angiogenesis (adapted from Nguyen *et al*, 2001; Bergers and Benjamin, 2003). The first step of angiogenesis involves the removal of pericytes or smooth muscle cells from the vessel. In response to a pro-angiogenic stimulus, such as VEGF, endothelial cells release proteases that degrade the basement membrane allowing endothelial cell migration towards the signal along with cell proliferation. Tubes are formed when the migrated endothelial cells develop a lumen and functional vessels occur with the recruitment of pericyte or smooth muscle cells which enhance the stability of the vessel.

1.1.4 Activators of Angiogenesis

There are a plethora of pro-angiogenic molecules (Table 1.1) that act directly on endothelial cells or on other target cells to initiate and sustain angiogenesis (Liekens *et al*, 2001). Of particular interest is VEGF which is a potent inducer of angiogenesis (Ferrara *et al*, 2003) and for that reason it was selected as a stimulator of angiogenesis in our experiments and will be more extensively reviewed here.

1.1.4a The Vascular Endothelial Growth Factor Protein Family

VEGF represents a protein family that includes several proteins such as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, svVEGF (snake venom VEGF) and PlGF (placental growth factor). They vary in their tissue distribution, interaction with receptor tyrosine kinases and in their overall effect during development and processes such as angiogenesis. Of particular interest is VEGF-A which acts on endothelial cells and is implicated in both physiological and pathological angiogenesis (Takahashi and Shibuya, 2005).

1.1.4a(i) VEGF-A

VEGF-A, located at chromosomal position 6p21.3, is composed of 7 introns and 8 exons. Alternative splicing generates nine isoforms (Fig. 1.1.2) including; VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{162}, VEGF-A_{165}, VEGF-A_{183}, VEGF-$A_{189,}$ VEGF-A_{206} and also the anti-angiogenic isoform VEGF-A_{165b} which competes for binding to the receptor. The isoform number denotes the number of amino acids (Ferrara *et al*, 2003). VEGF-A_{165} is classed as the predominant isoform (Liekens *et al*, 2001). This splice variant is the result of the removal of residues within exon 6 (Fig. 1.1.2) (Ferrara *et al*, 2003). It binds to and signals through the VEGF receptor tyrosine kinases VEGFR-1 and VEGFR-2 (Takahashi and Shibuya, 2005). In addition to its pro-angiogenic activity, $VEGF_{165}$ has also been reported to increase vascular permeability, induce vessel dilation and act as an inhibitor of apoptosis (Ferrara *et al*, 2003).

In embryonic stem cells, inactivation of one or both alleles of VEGF-A, results in abnormal blood vessel development and embryonic fatality at approximately 10.5 days after coitum respectively. Loss of VEGF prevents correct vasculogenesis, angiogenesis and lumen formation (Carmeliet *et al*, 1996). Likewise, Ferrara *et al*, 1996 reported heterozygous knockout of VEGF-A resulted in embryonic lethality around days 11-12 with an increase in apoptosis at day E11.5, insufficient blood supply to the yolk sak and abnormalities in development of the brain, heart and vasculature of other organs and tissues (Ferrara *et al*, 1996).

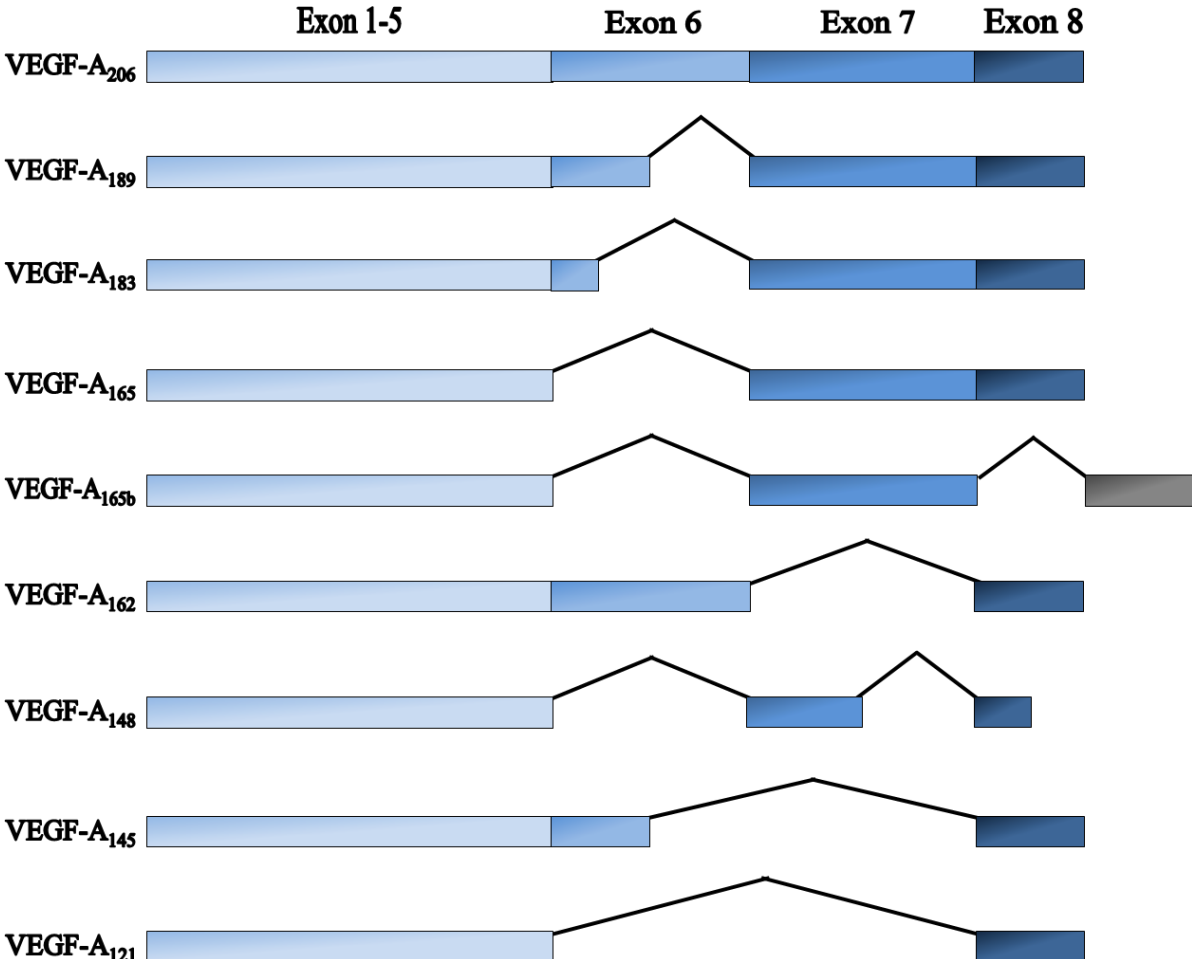

Exon 1-5
Exon 6
Exon 7
Exon 8
VEGF-A206
VEGF-A189
VEGF-A183
VEGF-A165
VEGF-A165b
VEGF-A162
VEGF-A148
VEGF-A145
VEGF-A121

Fig.1.1.2 Schematic diagram of the VEGF-A splice variants (adapted from Takahashi and Shibuya, 2005). VEGF-A is composed of seven introns and eight exons. Alternative splicing of this primary transcript generates nine isoforms; VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{162}, VEGF-A_{165}, VEGF-A_{183}, VEGF-A_{189}, VEGF-A_{206} and also the anti-angiogenic isoform VEGF-A_{165b}. The predominant isoform and the one implicated in the stimulation of angiogenesis is VEGF-A_{165} which has exon 6 removed.

1.1.4b VEGF Receptors

The VEGF receptors (VEGFR) are predominantly found on endothelial cells (Takahashi and Shibuya, 2005). Two main receptors are involved in VEGF-A signalling; VEGFR-1(Flt-1) and VEGFR-2 (Flk-1/KDR in mouse and human respectively) whose structures consist of an extracellular domain containing seven immunoglobulin (Ig) like domains, one transmembrane domain and a cytoplasmic tyrosine kinase domain (Takahashi and Shibuya, 2005; Ferrara *et al*, 2003). They are activated in response to VEGF binding which results in receptor dimerisation and auto-phosphorylation (Schlessinger, 2000).

1.1.4b(i) VEGFR-1 (Flt-1)

The VEGFR-1 is a 180 kDa protein. Interestingly, it has a higher affinity for VEGF-A by up to 10 fold compared to VEGFR-2 although VEGFR-2 is the main receptor involved in angiogenic processes (Takahashi and Shibuya, 2005). It is hypothesised that VEGFR-1 acts as a ‘decoy receptor’ preventing VEGF from binding and signalling through VEGFR-2 (Ferrara *et al*, 2003) although it has

been shown a monoclonal antibody against VEGFR-1 (KM1750) inhibits receptor binding to VEGF and down-regulates VEGF-induced migration of human umbilibal vein endothelial cells (HUVEC) (Kanno *et al*, 2000).

The role of VEGFR-1has been identified by use of knockout studies. Mutation of the Flt-1 gene in the mouse embryo does not affect the formation or proliferation of endothelial cells but does affect their ability to form functional channels resulting in embryonic death at roughly E8.5 days. The yolk sac contains no regulated vascular network and disrupted blood island structures are observed. Therefore this receptor is essential for vascularisation of the embryo (Fong *et al*, 1995).

1.1.4b(ii) VEGFR-2 (KDR/Flk-1)

The VEGFR-2 is a 200-230 kDa protein and is proposed to be the main receptor involved in VEGF-induced angiogenesis (Takahashi and Shibuya, 2005). Binding of VEGF-A to VEGFR-2 results in migration of HUVEC, DNA synthesis and extracellular signal-related kinase (Erk) activation (Kanno *et al*, 2000).

Interestingly, in the case of the knockout of Flk-1 a similar phenotype was observed to that seen for Flt-1 in that there was no organised blood vessels in the yolk sac or blood island formation and embryonic death occurred between days E8.5-9.5. Additionally, an increase in necrosis is observed and endothelial cells do not form correctly. Therefore, Flk-1 is also essential for vascularisation of the embryo in early development (Shalaby *et al*, 1995).

1.1.5 Anti-Angiogenic Therapies

Given the relevance of VEGF in the angiogenic processes associated to human diseases, it is not surprising that several therapeutic strategies have been developed to suppress the action of VEGF on these pathologies. This can be the result of preventing the VEGF/VEGFR interaction or by attenuating receptor activation. All have potential therapeutic benefits but are also associated with adverse effects on the patient (Gotink and Verheul, 2010; Kamba and McDonald, 2007; Chang *et al*, 2012) (Table 1.2. Summary of current anti-angiogenic treatments).

1.1.5a VEGF Inhibitors

1.1.5a(i) Bevacizumab (Avastin®)

Bevacizumab was the first anti-angiogenic drug to be used therapeutically (Shih and Lindley, 2006). It was approved by the Food and Drug Administration (FDA) in 2004 for metastatic colorectal cancer treatment. It is a humanised monoclonal antibody and has a half-life of 17-21 days (Ferrara *et al*, 2004). It binds to all isoforms of VEGF-A and inhibits its activity by preventing VEGF interacting with its receptors, VEGFR-1 and VEGFR-2 (Shih and Lindley, 2006). Bevacizumab has been associated with several side effects including impaired wound healing and proteinuria, with the most severe side effect being hypertension (Shih and Lindley, 2006; Hurwitz *et al*, 2004). Hypertension is thought to be the result of attenuation of nitric oxide (NO) production via endothelial nitric oxide synthase (eNOS) due to VEGF inhibition causing vasoconstriction and consequently an increase in blood pressure (Kamba and McDonald, 2007). It has been proposed the long half-life of bevacizumab may potentiate the adverse side effects associated with this therapy (Pieramici and Rabena, 2008). Treatment of

patients presenting rectal carcinomas using bevacizumab combined with 5-fluorouracil and radiation therapy resulted in a reduction in blood volume, interstitial fluid pressure and micro-vascular density of tumours (Willet *et al*, 2004). Co-treatment of bevacizumab with the chemotherapeutic agents irinotecan, fluorouracil and leucovorin (IFL) increased the median survival time of patients with metastatic colorectal cancer by up to 4.7 months compared to IFL treatment alone (Hurwitz *et al*, 2004).

1.1.5a(ii) Pegaptanib (Macugen®, EYE001, NX1838)

Pegaptanib is an RNA aptamer composed of 28 bases (Zhou and Wang, 2006) that binds and inhibits the function of VEGF-A_{165} by preventing its interaction with VEGFR-2 (Pieramici and Rabena, 2008). It was approved by the FDA in 2004 for the treatment of wet AMD. This inhibitor is engineered to bind VEGF-A_{165} with high affinity (Zhou and Wang, 2006) and is more stable due to resilience against nucleases (Gragoudas *et al*, 2004). Interestingly, in a rat model of proliferative retinopathy, pegaptanib treatment which inhibits rat VEGF-A_{164} (the equivalent isoform to human VEGF-A_{165}) attenuated pathological but not physiological neovascularisation, whereas inhibition of all VEGF isoforms reduced neovascularisation in both cases highlighting an advantageous role of isoform specific inhibition (Ishida *et al*, 2003). Pegaptanib treatment in patients displaying AMD resulted in reduced vessel permeability, reduced leakage, neovascularisation and a decrease in the size of the lesion (Gragoudas *et al*, 2004). Additionally, pegaptanib attenuated the loss of visual acuity and in some cases maintained or increased the visual acuity of patients

with AMD compared to those receiving sham injections (Gragoudas *et al*, 2004). It has been proposed its specificity for VEGF-A_{165} compared to all VEGF-A isoforms results in reduced efficacy of this treatment (Pieramici and Rabena, 2008) although side effects associated to this drug are highly reduced (Zhou and Wang, 2006). In fact, the majority of adverse effects are associated with the injection process rather than the drug itself, for example endophthalmitis, an intraocular infection that can lead to vision loss, occurred in a number of patients (Gragoudas *et al*, 2004).

1.1.5a(iii) Ranibizumab (Lucentis®)

Ranibizumab is a 48 kDa humanised monoclonal antibody also used for the treatment of AMD that was approved by the FDA in 2006. Unlike pegaptanib it binds all isoforms of VEGF-A, inhibiting their activity by blocking the VEGF/VEGFR interaction and the initiation of intracellular signalling (Kourlas and Abrams, 2007). It is generated using the Fab fragment from bevacizumab and has an approximately 20 times increase in VEGF binding affinity compared to bevacizumab (Pieramici and Rabena, 2008). Additionally, ranibizumab is smaller in size compared to bevacizumab and therefore penetrates better in tissue (Pieramici and Rabena, 2008). Clinical trials of ranibizumab for the treatment of neovascular AMD resulted in a reduction of the loss of visual acuity compared to the sham treated control patients. Interestingly, visual acuity was shown to be increased in patients treated with ranibizumab for 1 year which was associated with reduced growth and leakage from choroidal neovascularisation.

Few adverse effects were observed in the ranibizumab treated group compared to sham with the most severe being endophthalmitis (Rosenfeld *et al*, 2006).

1.1.5a(iv) VEGF-Trap

VEGF-Trap also acts as an inhibitor of VEGF by preventing its interaction with VEGFR. It has the highest affinity for VEGF out of the inhibitors (Chang *et al*, 2012; Holash *et al*, 2002) as it is composed of the second and third Ig domains of VEGFR-1 and VEGFR-2 respectively (which are the regions of the receptor involved in VEGF binding in each case) combined with a human IgG Fc fragment and therefore acts as a decoy receptor (Holash *et al*, 2002). Due to its small size it is proposed to improve its penetration in tissues (Pieramici and Rabena, 2008). It interacts with all isoforms of VEGF-A and additionally placental growth factor (PlGF) -1 and 2, suggesting it has the potential to be a strong anti-angiogenic molecule by inhibiting two stimulators of angiogenesis. Additionally, VEGF-Trap has been shown to have a long half-life after injection into the eye (Chang *et al*, 2012). VEGF- Trap treatment in patients with neovascular AMD results in reduced lesion size and choroidal neovascularisation and significantly improves visual acuity. Adverse effects observed are conjunctival hemorrhage associated with the injection procedure and increases in intraocular pressure and uveitis. Systemic side effects such as bronchitis and infection of the upper respiratory and urinary tract were also reported (Heier *et al*, 2011). It is proposed the high affinity of VEGF-Trap for VEGF will result advantageously in longer intervals between required treatments and therefore may reduce adverse effects associated with the administration of this treatment (Heier *et al*, 2011).

1.1.5b VEGFR Inhibitors

VEGFR inhibitors can either be monoclonal antibodies (Casanovas *et al*, 2005) which bind to the surface of the receptor or receptor tyrosine kinase inhibitors such as small molecule inhibitors, which can pass through the membrane and bind to cytosolic regions of the receptor, either at the ATP binding site or near to it, preventing both ATP binding and activation of signalling (Gotink and Verheul, 2010).

1.1.5b(i) Sunitinib (Sutent®, SU11248)

Sunitinib was FDA approved in 2006 (Faivre *et al*, 2007). It is a small molecule inhibitor that binds directly to the ATP binding site inhibiting ATP interaction and preventing receptor activation (Gotink and Verheul, 2010). It inhibits VEGFR-1,2 and 3 and additionally the platelet-derived growth factor receptor (PDGFR) (Faivre *et al*, 2007). Administration of sunitinib has been reported to reduce significantly the growth and microvessel density of several kinds of tumours in animal models of cancer or clinical trials of human patients (Mendel *et al*, 2003; Motzer *et al*, 2006). Unfortunately, this molecule is associated with a number of side effects including, diarrhea, vomiting, fatigue, hypertension and hand-foot syndrome (delicate skin on the hands and feet that can blister and peel in severe cases) although it was reported these were manageable and ended upon completion of treatment (Faivre *et al*, 2007). Additionally, due to being a small molecule inhibitor it has a short half-life and therefore it is proposed the rate of recovery of adverse effects will be quicker (Kamba and McDonald, 2007).

1.1.5b(ii) Sorafenib (Nexavar®, Bay 43-9006)

Sorafenib, approved in 2005 by the FDA for the treatment of renal cell carcinoma (RCC) (Wilhelm *et al*, 2006), is a small molecule inhibitor that binds to the intracellular region of the VEGFR. It binds at a site next to the ATP binding site altering its conformation and preventing receptor interaction with ATP and signal transduction activation (Gotink and Verheul, 2010; Wilhelm *et al*, 2006). As in the case of other receptor inhibitors, animal models and clinical trials in human patients have demonstrated the efficiency of sorafenib in reduction of tumour growth and microvessel density (Wilhelm *et al*, 2004; Ratain *et al*, 2006). Side effects were observed such as fatigue, hand-foot syndrome and diarrhea which could be alleviated by dose reduction. The most severe side effect was hypertension which could be controlled by the use of anti-hypertensive drugs (Ratain *et al*, 2006). As sorafenib is also a small molecule inhibitor it is suggested the recovery rate of adverse effects will be faster due to a shorter half-life as proposed for sunitinib (Kamba and McDonald, 2007).

1.1.5b(iii) IMC-1121b (Ramucirumab)

The monoclonal antibody ramucirumab is an anti-VEGFR antibody. It binds to VEGFR-2 preventing all isoforms of VEGF interacting with this receptor (Spratlin *et al*, 2010). Ramucirumab treatment of patients with advanced solid malignancies resulted in reduced perfusion and vascularity of tumours although adverse effects such as hypertension, proteinuria, abdominal pain, headaches, deep vein thrombosis (DVT) and vomiting were observed (Spratlin *et al*, 2010).

Anti-Angiogenic Treatments	Composition	Mechanism of Action	Advantages	Disadvantages
Bevacizumab (Avastin®)	Humanised monoclonal antibody	Binds all isoforms of VEGF-A. Inhibits VEGF/VEGFR interaction	-Increase in median survival time (4.7 months) when used as a treatment for metastatic colorectal cancer	-Impaired wound healing, proteinuria, hypertension Longer half- life therefore potential for increased side effects
Pegaptanib (Macugen®)	RNA Aptamer	Inhibits binding of VEGF-A_{165} to VEGFR2	-Reduced adverse effects as specifically targets VEGF-A_{165} - Increased stability	-Potential reduced efficacy as only targets one VEGF-A isoform
Ranibizumab (Lucentis®)	Humanised monoclonal antibody Fab	Binds all isoforms of VEGF-A. Inhibits VEGF/VEGFR interaction	-Smaller therefore penetrates better in tissue -Higher binding affinity to VEGF than bevacizumab - Few adverse effects	-Endophthalmitis observed in treatment of AMD
VEGF-Trap	Second and third Ig domains of VEGFR-1 and VEGFR-2 respectively combined with a human IgG Fc fragment	Acts as a decoy receptor. Interacts with all VEGF-A isoforms and PlGF-1 and 2, inhibiting their interaction with their corresponding receptors	-Highest affinity out of all anti-angiogenic treatments, therefore potential for increased interval between treatments -Interacts with other pro-angiogenic factors therefore may enhance anti-angiogenic effect -Smaller than bevacizumab therefore improved penetration in tissue	-Conjunctival hemorrhage increase in intraocular pressure, uveitis, bronchitis, infection of the upper respiratory and urinary tract
Sunitinib (Sutent®)	Small molecule inhibitor	Binds to ATP binding site of VEGFR-1, 2, 3, PDGFR preventing its activation	-Short half-life therefore faster recovery rate of adverse effects	-Diarrhea, vomiting, fatigue, hypertension, hand-foot syndrome
Sorafenib (Nexavar®)	Small molecule inhibitor	Binds to VEGFR. Prevents its activation by ATP	-Short half-life therefore faster recovery rate of adverse effects	-Hypertension, fatigue, hand-foot syndrome, diarrhea
IMC-1121B (Ramucirumab)	Monoclonal antibody	Binds to VEGFR-2 preventing VEGF (all isoforms)/VEGFR2 interaction	-Prevents VEGFR-2 interaction with all isoforms of VEGF therefore may enhance anti-angiogenic potential	-Hypertension, proteinuria, vomiting, headache, abdominal pain, deep vein thrombosis

Table 1.2 Summary of Current Anti-Angiogenic Treatments

1.1.6 Problems Derived From Current Anti-Angiogenic Therapies

1.1.6a Resistance to Angiogenic Inhibitors

It was initially thought that anti-angiogenic inhibitors would result in reduced resistance to treatment as they act on the genetically stable endothelial cells rather than tumour cells which can readily mutate and adapt to therapy (Rosen, 2000). However, it has been shown in a mouse model of islet cell carcinogenesis, prolonged inhibiton of VEGFR-2 signalling results in an increase in the expression of pro-angiogenic proteins such as fibroblast growth factor (FGF) and loss of intital regression of tumour growth suggesting resistance to angiogenic inhibitors can occur due to an increase in pro-angiogenic molecules and signalling via alternative pathways (Casanovas *et al*, 2005). Additionally, some treatments may lead to an increase in expression of both VEGF and its receptors, therefore upon the termination of the anti-angiogenic treatment, tumour growth can reoccur as angiogenesis will be activated once again (Verheul and Pinedo, 2007).

1.1.6b Combined Therapy

Originally chemotherapy and radiotherapy were the main treatments for tumours. However, severe side effects were seen with these treatments as they do not differentiate between tumoural and non-tumoural cells (Wilhelm *et al*, 2006). Due to this, combination therapy of these treatments with anti-angiogenic compounds was proposed. It is hypothesised drug delivery to the tumour will be improved in the presence of angiogenic inhibitors as the interstitial pressure within the tumour will be reduced and the vasculature will return to a normalized state that is no longer permeable. Both the tumoural cells and the endothelial cells that respond to the pro-angiogenic signals released from the tumour are targeted in this form of treatment by chemotherapy/radiotherapy and anti-angiogenic compounds respectively (Jain, 2001). The benefit of combined

therapy has now been shown in the treatment of metastatic colorectal cancer. In patients treated with the IFL, the addition of bevacizumab resulted in an increase in the median overall survival, the median length of progression free survival and the one year survival rate which correlated with a reduction of up to 34% of the likelihood of death compared to the placebo group (Hurwitz *et al*, 2004).

1.1.7 VEGF Signalling Pathways in Angiogenesis

VEGF signals through a variety of cellular pathways involved in various aspects of angiogenic processes and is therefore a very diverse stimulus (Fig. 1.1.3) (Takahashi and Shibuya, 2005).

1.1.7a p38 Mitogen-Activated Protein Kinase (MAPK)

There are four isoforms of the p38 MAPK including β, α, γ and δ which belong to the MAPK family. This protein becomes activated when phosphorylated by MAP/Erk Kinase Kinase (MEK) 3 and 6 (McMullen *et al*, 2005). The predominant isoform activated in endothelial cells in response to MEK6 activation was found to be p38α (McMullen *et al*, 2005). Furthermore, p38 MAPK phosphorylation and activation was shown to be upregulated in HUVEC in response to VEGF-stimulation and its activity was shown to inhibit endothelial cell proliferation (Yu and Sato, 1999) via inhibiton of Erk activity and increased cell migration via activation of its substrate, heat shock protein 27 (Hsp27) (Fig. 1.1.3) (McMullen *et al*, 2005).

1.1.7b Extracellular Signal-Related Kinase (Erk) Pathway

Erk1/2 is a serine/threonine kinase also known as p44/p42 MAPK. This signalling pathway is a kinase cascade (Meloche and Pouysségur, 2007). Initially, Ras is activated by receptor stimulation and phosphorylates the MAP kinase kinase kinase (MAPKKK) Raf. Raf then activates the MAP kinase kinase (MAPKK) MEK1/2 which consequently phosphorylates threonine and tyrosine residues on the MAP kinase (MAPK) Erk1/2 and increases its activity (Meloche and Pouysségur, 2007). Specifically, phosphorylation and activation of Erk1/2 by VEGF has been reported to promote HUVEC proliferation (Yu and Sato, 1999; Meadows *et al*, 2001) and cell survival of Human Dermal Microvascular Endothelial Cells (HDMEC) by preventing apoptosis (Fig. 1.1.3) (Gupta *et al*, 1999).

1.1.7c Phosphatidylinositol-3 Kinase (PI3K)

The PI3K pathway is involved in the activation of p70 S6 kinase and consequently the proliferation of HUVEC in response to VEGF-signalling (Yu and Sato, 1999). Additonally, the serine/threonine kinase Akt is also activated by PI3K in VEGF-induced HUVEC which enhances cell survival (Fujio and Walsh, 1999) (Fig. 1.1.3).

1.1.7d Phospholipase C-γ (PLC-γ)

VEGF stimulation of VEGFR-2 activates PLC-γ activity which in turn results in the production of Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) (Holmes *et al*, 2010). DAG and IP_3 activate the protein kinase C (PKC) (Holmes *et al*, 2010) and the calcineurin/nuclear factor of activated T-cells (NFAT) pathways respectively (Crabtree and Olson, 2002).

1.1.7d(i) Protein Kinase C (PKC)

Stimulation of bovine aortic endothelial cells (BAEC) with VEGF has been reported to increase PKC activity which is accompanied by a shift in its localisation from the cytosol to the membrane. VEGF-dependent activation of PKC signalling has been shown to play a role in endothelial cell proliferation and survival (Xia *et al*, 1996) (Fig. 1.1.3).

1.1.7d(ii) Calcineurin/NFAT

Calcineurin is a serine/threonine protein phosphatase that is activated by calmodulin in a calcium dependent manner (Li *et al*, 2011). Activated calcineurin consequently dephosphorylates NFAT resulting in its translocation from the cytoplasm to the nucleus where it binds to DNA and activates the transcription of target genes (Crabtree and Olson, 2002). The calcineurin/NFAT pathway has been shown to regulate angiogenesis in endothelial cells in response to VEGF-stimulation (Hernández *et al*, 2001) (Fig. 1.1.3) and will be discussed extensively in section 1.2.6.

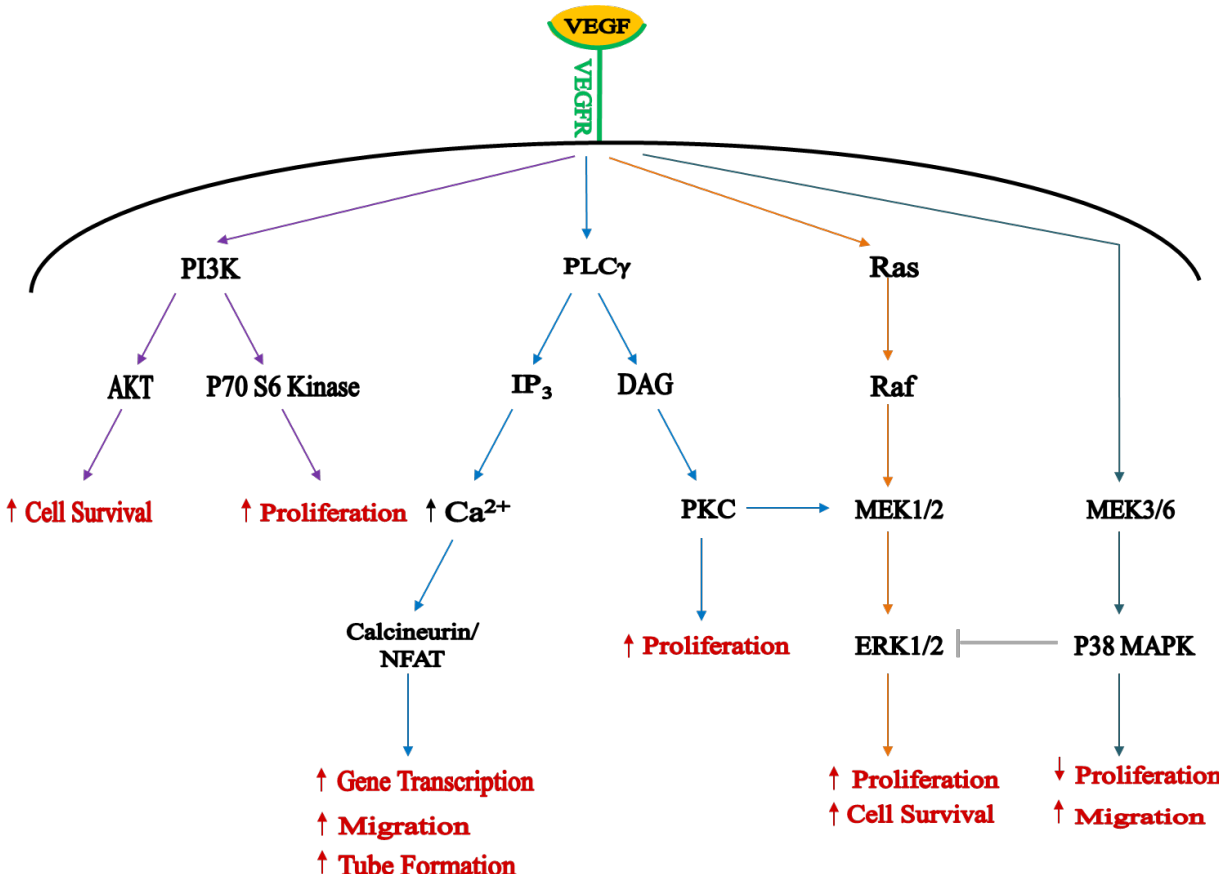


Fig. 1.1.3 VEGF-induced signalling pathways involved in angiogenesis (adapted from Takahashi and Shibuya, 2005). VEGF/VEGFR interaction stimulates a variety of signalling pathways required for successful angiogenesis including the phosphatidylinositol-3 kinase (PI3K) pathway that activates both Akt and P70 S6 kinase required for cell survival and proliferation respectively. The phospholipase C-γ (PLCγ) pathway activates the production of Inositol 1,4,5-trisphosphate (IP_3) and Diacylglycerol (DAG) which stimulate the calcineurin/nuclear factor of activated T cell (NFAT) pathway and protein kinase C (PKC) pathway respectively consequently increasing gene transcription and proliferation. Erk1/2, required for cell proliferation, is activated via the Ras/Raf/MEK pathway is also activated by PKC but is inhibited by p38 mitogen activated protein kinase (p38MAPK). P38MAPK as well as inhibiting cell proliferation has been reported to increase cell migration.

1.2 The Calcineurin/NFAT Pathway

The calcineurin/NFAT pathway converts calcium signalling into the expression of target genes (Li *et al*, 2011) which are required in a variety of cellular events such as the immune response (Crabtree and Olson, 2002) and angiogenesis (Iizuka *et al*, 2004; Hernández *et al*, 2001). Specifically, activation of this signalling pathway in HUVEC has been shown to occur in response to VEGF-signalling (Armesilla *et al*, 1999).

1.2.1 Calcineurin

Calcineurin (Cn), also referred to as protein phosphatase2B (PP-2B), is a serine/threonine phosphatase of approximately 80 kDa (Klee *et al*, 1998) that has been shown to have an important role in diverse biological processes such as regulation of the immune response (Clipstone and Crabtree, 1992), apoptosis (Klee *et al*, 1998) and angiogenesis (Hernández *et al*, 2001).

Calcineurin is a heterodimer composed of a catalytic A and a regulatory B subunit of approximately 60 kDa and 20 kDa respectively. The A subunit is further subdivided into a catalytic domain and a regulatory region which is composed of a calcineurin B binding region, calmodulin binding domain and an auto-inhibitory domain running from N-terminal to C-terminal respectively (Fig. 1.2.1) (Li *et al*, 2011).

Calcineurin A (Cn A) is encoded by three genes which generates three isoforms; Cn Aα, Cn Aβ and Cn Aγ. They vary in their tissue distribution with Aα and Aβ isoforms being present in the brain and lympocytes while Cn Aγ is tissue specific being localised to the testis (Matrínez-Martínez and Redondo, 2004). In comparison, Cn B is encoded by two genes (Graef *et al*, 2001) generating the isoforms Cn B1 and Cn B2, with isoform B1 being ubiquitously

expressed whereas B2 expression is restricted to the testis (Musson *et al*, 2012).

Calcineurin is activated by both calcium and calcium/calmodulin (Li *et al*, 2011). The regulatory B subunit of calcineurin contains four EF hand motifs; two with high affinity and two with low affinity for calcium (Fig. 1.2.1). The high affinity sites are generally constantly occupied by a calcium ion and have been suggested to have a role in the maintenance of the structure of calcineurin. In comparison the low affinity sites acts as sensors for the concentration of calcium in the surrounding environment (Li *et al*, 2011). When the calcium concentration increases, the low affinity calcium sites become occupied and calcineurin is partially activated. These low affinity sites also induce a conformational change in the calcineurin A subunit that opens up the calmodulin binding domain (Yang and Klee, 2000). Calmodulin binding is proposed to displace the auto-inhibitory domain (Tokoyoda *et al*, 2000) and increases the activity of calcineurin up to 20 fold (Klee *et al*, 1998).

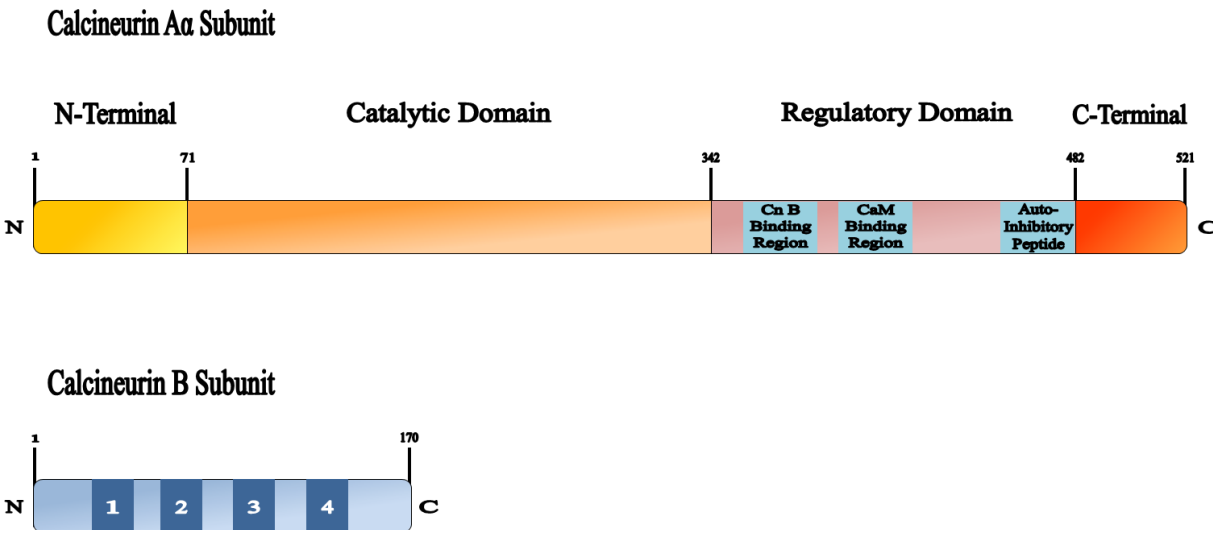


Fig. 1.2.1 Structure of the human calcineurin A and B subunits (adapted from Li *et al*, 2011; Klee *et al*, 1998). Calcineurin is composed of a catalytic A and regulatory B subunit. The A subunit (numbered according to human calcineurin Aα) is divided into four regions; the N-terminal region, the catalytic domain, the regulatory domain and the C-terminal region. The regulatory domain of subunit A contains the calcineurin B (Cn B) binding region which interacts with the calcineurin B subunit, the calmodulin binding region which interacts with calmodulin resulting in activation of calcineurin upon displacement of the auto-inhibitory peptide that is also found in this region. The calcineurin B subunit contains 4 sites for calcium binding numbered 1-4. Two of these have high affinity for calcium and the other two have low affinity and act as sensors for calcium concentration in the surrounding environment.

1.2.2 Calcineurin Knockout and Mutational Studies

Calcineurin $A\alpha^{-/-}$ mice have revealed a role for calcineurin in T-cell signalling and therefore the immune response. T-cell proliferation in response to antigen stimulation in knockout mice was reduced whereas proliferation in response to mitogen stimulation, such as Phorbol-12 Myristate 13-Acetate (PMA) and ionomycin was unaffected. Additionally, *in vitro* re-stimulation of T-cells from knockout mice secreted less IL-2, IL-4 and IFN-γ than the wild-type. Interestingly, NFAT nuclear localisation was unaffected in response to mitogen stimulation (Zhang *et al*, 1996). In comparison, calcineurin $A\beta^{-/-}$ mice have reduced translocation of NFAT to the nucleus compared to the wild-type. Loss of Cn Aβ also resulted in a decrease in the proliferation and number of T-cells. The production of cytokines such as IL-4, IFN-γ and IL-2 was also down-regulated in response to mitogen stimulation in Cn $A\beta^{-/-}$ mice (Bueno *et al*, 2002).

Mutation of the Cn B gene in mice prevents activation of Cn A and consequently leads to embryonic lethality at days E10.5-E11.5. Calcineurin signalling was shown to be required between days E7.5-E8.5. In Cn $B^{-/-}$ mice, initial vascularisation occurs but fails to develop into organised structures to that seen in the wild-type. Angiogenesis of the yolk sac is reduced in addition to a decrease in the recruitment of smooth muscle cells to the aorta. Furthermore, VEGF-A was shown to be over-expressed in Cn $B^{-/-}$ embryos. Therefore, Cn B is required for the correct patterning of the vasculature which is proposed to occur through negative-regulation of VEGF-A expression (Graef *et al*, 2001).

Moreover, B-cell specific knockout of Cn B1 inhibits dephosphorylation of NFAT, in response to PMA and Ionomycin stimulation, and consequently calcineurin activity which downregulates the transcription of NFAT-dependent target genes. Cn $B1^{-/-}$ B-cells were also shown to have reduced proliferation and increased IgM levels in response to T-cell-independent antigen signalling compared to the wild-type (Winslow *et al*, 2006).

1.2.3 Nuclear Factor of Activated T Cells (NFAT)

NFAT is an approximately 120 kDa protein (Ruff and Leach, 1995) that is activated by calcineurin and initiates the transcription of target genes (Crabtree and Olson, 2002) such as those required for angiogenesis (Iizuka *et al*, 2004; Hernández *et al*, 2001).

NFAT consists of a transactivation domain, a NFAT homology region (NHR) (also referred to as the regulatory region), a DNA binding domain (also termed the Rel homology region (RHR)) and a C-terminal domain, running from N-terminal to C-terminal respectively (Fig. 1.2.2) (Mancini and Toker, 2009; Li *et al*, 2011). Both the NHR and DNA binding domain, consist of approximately 300 residues each and are conserved among NFAT isoforms (Rao *et* al, 1997). Within the NHR of NFAT, three conserved serine/proline (SP) repeats (SP1, SP2 and SP3) (Mancini and Toker, 2009; Hogan *et al*, 2003) which each have the sequence, SPXXSPXXSPXXXXXD/ED/E, where X is any amino acid (Crabtree, 1999) and a conserved serine rich region (SRR1) are found (Beals *et al*, 1997). They contain the 13 serine residues which become dephosphorylated in response to calcineurin activation which is required for NFAT localisation to the nucleus (Fig. 1.2.2) (Hogan *et al*, 2003; Beals *et al*, 1997).

There are five main isoforms of human NFAT that are ubiquitously expressed (additional nomenclature that each isoform is known as is shown in brackets); NFAT1 (NFATc2, NFATp), NFAT2 (NFATc1, NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3, NFATx) and NFAT5 (tonicity-responsive enhancer-binding protein (TonEBP) and osmotic response element binding protein (OREBP)). Of particular interest is isoforms NFAT1-4 which are regulated by calcium (Mancini and Toker, 2009).

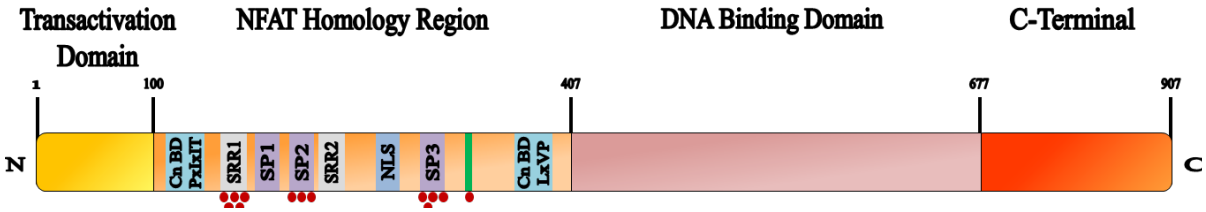


Fig. 1.2.2 Schematic diagram of the structure of NFAT and its domains (adapted from Mancini and Toker, 2009; Li *et al*, 2011; Rao *et al*, 1997). NFAT is composed of four domains; the transactivation domain, the NFAT homology region (NHR) (also known as the regulatory region), the DNA binding domain (also known as the Rel homology domain) and the C-terminal region. Both the NHR and the DNA binding domain are highly conserved amongst the isoforms. The NHR contains two calcineurin binding domains (Cn BD) PxIxIT and LxVP at its N and C-terminals respectively. Two serine rich regions (SRR) and three serine/proline (SP) repeats are also present here and are highly conserved. These contain 13 serine residues (represented as red circles) which become dephosphorylated by calcineurin to allow NFAT localisation to the nucleus and activation of the transcription of target genes. Two nuclear localisation sequences (NLS) are also present, one in the NHR and one in the DNA binding domain which become exposed in response to dephosphorylation of serine residues by calcineurin.

1.2.4 NFAT Knockouts

1.2.4a NFAT1

NFAT1$^{-/-}$ mice have a decrease in the cross sectional area of myofibers, but not the overall number, resulting in a reduced muscle size compared to the wild-type, demonstrating a role for NFAT1 in the regulation and growth of skeletal muscle (Horsley *et al*, 2001).

Additionally, NFAT1 is involved in the regulation of the immune response. NFAT1$^{-/-}$ mice have splenomegaly and hyperproliferation of both B and T-cells due to a reduction of CD40 and Fas ligand. The production of IL-4 is also reduced at early time points but increased at later time points compared to the wild-type (Hodge *et al*, 1996).

1.2.4b NFAT2

NFAT2 has been implicated in the regulation of heart development specifically in valve formation. NFAT2$^{-/-}$ mice are embryonic lethal at E14.5 days due to lack of development of the cardiac valves and septa but begin to show cardiac defects around E12.5, such as a reduction in chamber size in addition to the aortic sac having both thicker walls and a thinner lumen compared to the wild-type. Additionally, at E13.5 slower development of semilunar valves is observed (De la Pompa *et al*, 1998). A similar result was seen in a study by Ranger et al, 1998 that also studied NFAT2$^{-/-}$ mice. Congestive heart failure caused embryonic lethality between days E13.5-17.5 as a result of the loss of aortic and pulmonary valve formation (Ranger *et al*, 1998a).

1.2.4c NFAT3

Disruption of the NFAT3 gene to generate NFAT3$^{-/-}$ knockout mice had no effect on embryonic development. However, when this was combined with NFAT4$^{-/-}$ mice, embryonic development defects began to be observed around E10.5 days, such as a less vascularised yolk sac and development compared to the wild-type, with lethality occurring around E11.5 due to formation of dysfunctional vessels. These isoforms of NFAT are also required for recruitment of smooth muscle cells to the vessels to enhance stability (Graef *et al*, 2001).

1.2.4d NFAT4

NFAT4, as well as having a role in the stabilisation of the vasculature and functionality of vessels in the developing heart (see above) (Graef *et al*, 2001), has also been implicated in the regulation of skeletal muscle growth as at E17.5 days a smaller cross sectional area of muscle was observed in NFAT4$^{-/-}$ mice. A reduction in the size of both fast and slow muscles resulting in an overall decrease in muscle mass compared to wild-type was also seen and shown to be a consequence of a decrease in myofibre number as a result of abnormal development, leading to a reduced number of primary myofibres (Kegley *et al*, 2001).

Additionally, NFAT4$^{-/-}$ mice have a reduced number of mature single positive thymocytes which is thought to occur due to an increase in apoptosis of double positive thymocytes as a result of a decrease in Bcl-2 protein expression.

Therefore, NFAT4 has a protective role against apoptosis in the formation of single positive thymocytes (Oukka *et al*, 1998).

Transgenic mice containing a double knockout (DKO) of NFAT1 and 4 were shown to have a role in the negative regulation of the immune response. At seven weeks of age, DKO mice have an increase in mast and eosinophil cells in the spleen and lymph nodes and display spleenomegaly and lymphadenopathy which was thought to be the result of a 2-3 fold increase in proliferation of spleenocytes and lymph node cells. An increase in T-cell proliferation was also observed which was suggested to be the result of Fas ligand transcription inhibition. The cytokines IL-4, IL-5, IL-6 and IL-10 were also upregulated in DKO mice compared to the wild-type. It was proposed NFAT1 and 4 act as regulators of the threshold at which T-cells can be activated and in their absence this threshold is lower, resulting in greater T-cell activation and responses such as hyperproliferation (Ranger *et al*, 1998b).

1.2.5 Mechanism of NFAT Translocation From the Cytoplasm to the Nucleus

Calcineurin has been shown to be required for successful NFAT nuclear localisation and the consequent transcription of target genes (Beals *et al*, 1997; Shibasaki *et al*, 1996; Rao *et al*, 1997). Binding of a stimulus to a receptor such as VEGF to VEGFR initiates the PLC signalling pathway (Mancini and Toker, 2009) which stimulates the release of intracellular stores of calcium from the endoplasmic reticulum (ER) in response to IP_3 signalling (Fig. 1.2.3) (Crabtree and Olson, 2002). However, stimulation of NFAT for its nuclear translocation requires a prolonged calcium signal (Timmerman *et al*, 1996). The sustained

calcium levels in the cytosol are achieved by the activation of the calcium-release activated calcium channels (CRAC). The increase in cytosolic calcium results in calcium interaction with calmodulin and consequently initiates calcineurin activity (Mancini and Toker, 2009). Activated calcineurin interacts with the N-terminal region of NFAT (Luo *et al*, 1996) at two distinct regions, one N-terminal and one C-terminal, of the regulatory region of NFAT denoted PxIxIT and LxVP respectively (Fig. 1.2.2) (Li *et al*, 2011). PxIxIT is highly conserved among NFAT proteins whereas LxVP was originally identified in NFAT 2 and 4 only (Hogan *et al*, 2003).

Both the critical serine residues in the SP repeats and SRR have been shown to be essential for NFAT2 nuclear localisation. Two nuclear localisation sequences (NLS) between residues 265-267 (KRK) and 682-685 (KRKK) have also been reported to be involved in this process. It was shown calcineurin dephosphorylates the serine residues, which is proposed to expose the nuclear localisation sequences and induce concomitant NFAT nuclear translocation (Beals *et al*, 1997).

Interestingly, NFAT4 and calcineurin were shown to interact and additionally co-localise to the nucleus in response to ionophore stimulation. It has been proposed calcineurin binding to NFAT exposes the NLS and due to the interaction with NFAT co-translocates to the nucleus sustaining NFAT activity during calcium signalling (Fig. 1.2.3) (Shibasaki *et al*, 1996).

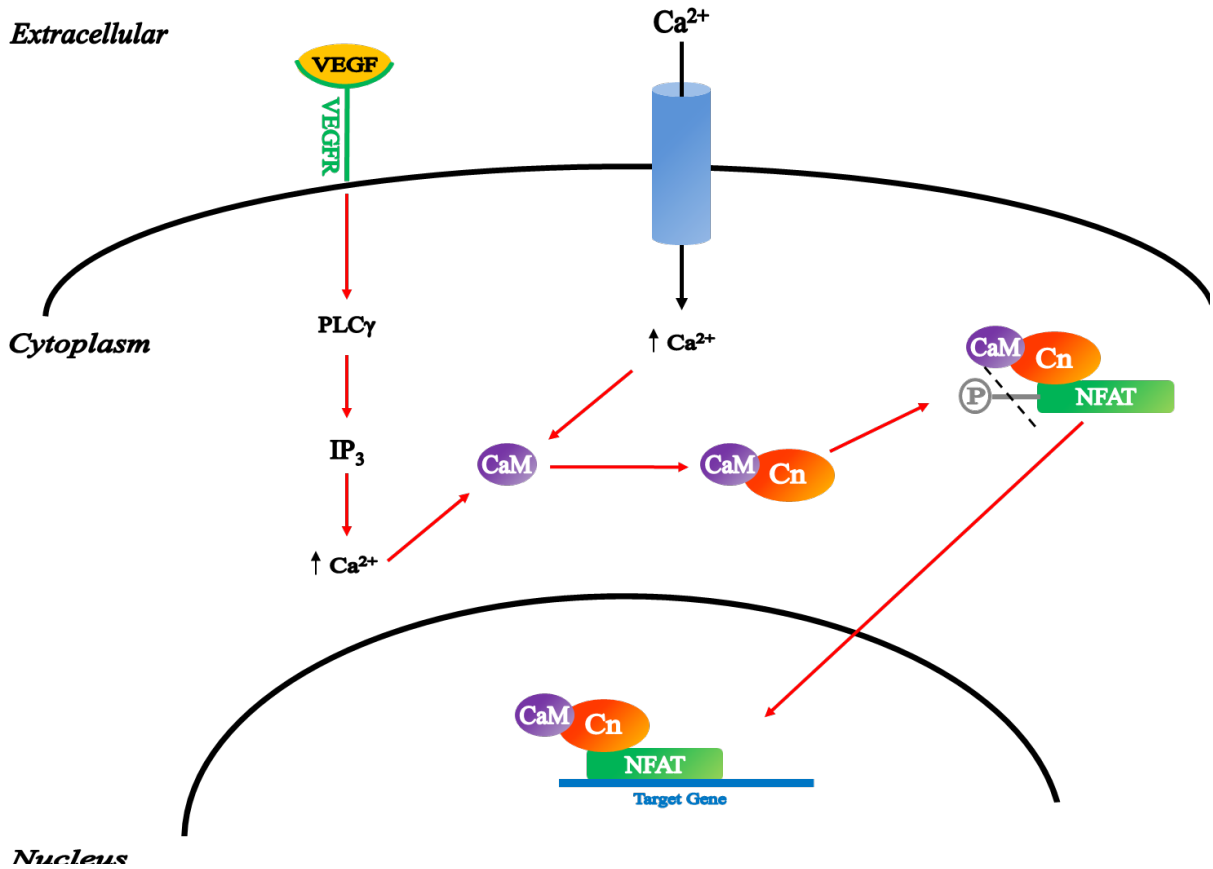


Fig. 1.2.3 Diagram of the calcineurin/NFAT signalling pathway (adapted from Mancini and Toker, 2009; Rao *et al*, 1997). In response to VEGF binding to its receptor, phospholipase C-γ (PLCγ) is activated and stimulates the production of inositol trisphosphate (IP_3). This increases the intracellular levels of calcium as it is released from the endoplasmic reticulum and stimulates the sustained increase in cytosolic calcium via opening of the calcium-release activated calcium channel (CRAC). This activates calmodulin (CaM) which binds to calcineurin (Cn) and initiates its binding to NFAT. This interaction results in dephopshoyrlation of critical serine

residues within NFAT which activates it and allows its translocation to the nucleus in conjunction with calcineurin. Once in the nucleus it binds to DNA.

1.2.6 The Calcineurin/NFAT Pathway in Biological Processes

The calcineurin/NFAT pathway has been implicated in biological processes such as skeletal and cardiac development, apoptosis and the immune response however these topics are out of the scope of this research (for a review see Crabtree and Olson, 2002; Klee *et al*, 1998). In this study we will focus on the role of the calcineurin/NFAT pathway in angiogenesis.

Successful angiogenesis requires, cell migration, tube formation and proliferation (Carmeliet, 2000; Liekens *et al*, 2001). A study by Hernández *et al*, 2001 identified a role for the calcineurin/NFAT pathway in angiogenesis as inhibition of calcineurin by Cyclosporin A (CsA) was shown to attenuate VEGF-induced endothelial cell migration, *in vitro* tubular morphogenesis and an *in vivo* mouse model of corneal angiogenesis. However, calcineurin did not regulate cellular proliferation in response to VEGF-signalling (Hernández *et al*, 2001).

Interestingly the expression of two pro-angiogenic proteins; regulator of calcineurin 1.4 (RCAN1.4) and Cyclooxygenase-2 (Cox-2) have been reported to be regulated by the calcineurin/NFAT pathway. RCAN1.4 and Cox-2 have been shown to contain 15 and 2 NFAT binding sites in their promoters respectively (Yang *et al*, 2000; Hernández *et al*, 2001). Additionally, VEGF stimulation of endothelial cells enhances their expression which is inhibited in the presence of CsA showing a calcineurin dependent mechanism of expression (Holmes *et al*, 2010; Iizuka *et al*, 2004; Hernández *et al*, 2001).

The pro-angiogenic proteins RCAN1.4 and Cox-2 have also been implicated in angiogenic processes (Iizuka *et al*, 2004; Hernández *et al*, 2001). For example, knockdown of RCAN1.4 has been shown to inhibit endothelial cell migration and tubular morphogenesis both *in vitro* and *in vivo* in response to VEGF-stimulation but has no effect on proliferation (Holmes *et al*, 2010; Iizuka *et al*, 2004). In contrast, other studies have demonstrated a negative role for RCAN1.4 proposing it to be a negative feedback inhibitor of calcineurin activity (see section 1.2.7e) (Yao and Duh, 2004) and consequently angiogenesis (Hesser *et al*, 2004). For instance, ectopic expression of RCAN attenuated the expression of the pro-angiogenic protein Cox-2. Over-expression of RCAN also attenuated *in vitro* and *in vivo* tube formation and inhibited tumour growth in mice. RCAN1.4 also down-regulated VEGF- induced proliferation (Hesser *et al*, 2004; Minami *et al*, 2004; Qin *et al*, 2006). The reason for this discrepancy in RCAN's role in the regulation of angiogenesis is still unclear.

Cox-2 has also been implicated in the regulation of angiogenesis as a variety of Cox-2 inhibitors have been shown to down-regulate PMA-induced migration and tube formation of endothelial cells (Woods *et al*, 2003; Daniel *et al*, 1999). Moreover, inhibition of Cox-2 by the inhibitor NS-398 resulted in attenuation of VEGF-induced *in vitro* and *in vivo* tube formation. Furthermore, the inhibition of angiogenesis by either CsA or NS-398 could be reversed with the addition of the Cox-2 product Prostaglandin E_2 (PGE_2) (Hernández *et al*, 2001). Interestingly, PGE_2 was shown to upregulate the expression of VEGF in endothelial cells (Pai *et al*, 2001). As previously mentioned, VEGF is a pro-angiogenic molecule (Takahashi and Shibuya, 2005) and therefore suggests Cox-2 is involved in the propagation of angiogenic signalling.

1.2.7 Inhibitors of the Calcineurin/NFAT Pathway

1.2.7a Cyclosporin A and FK506

Cyclosporin A (CsA) and tacrolimus (FK506) are two immunosuppressive drugs that interact with the intracellular proteins (immunophilins); cyclophilin A and FK506 binding protein (FKBP12) respectively (Li *et al*, 2011). The inhibitory complexes formed bind to the border between the calcineurin A and B subunits at similar sites (Klee *et al*, 1998) preventing its interaction with NFAT, inhibiting NFAT dephosphorylation and consequently attenuating its activation (Ruff and Leach, 1995; Loh *et al*, 1996; Fruman *et al*, 1992). These drugs have been used in transplant therapy to prevent transplant rejection (Crabtree and Olson, 2002) due to inhibition of NFAT activity and consequently reduction of gene transcription in immune cells such as T-cells (Li *et al*, 2011). Additionally, they were tested for the treatment of cardiac hypertrophy. Unfortunately the dosage required for treatment highly exceeds the amount needed to subdue the immune response (Crabtree, 1999) and treatment was associated with substantial side effects including kidney damage, renal failure, hypertension, diabetes (hyperglycaemia) and gastrointestinal problems (Matrínez-Martínez and Redondo, 2004).

1.2.7b Cabin 1/Cain

Cabin1 (calcineurin binding)/ cain (calcineurin inhibitor) is an approximately 230 kDa protein that interacts with calcineurin and inhibits its activity (Lai *et al*, 1998;

Sun *et al*, 1998). At the mRNA and protein level it was shown to be localised predominantly in the brain and liver. Interestingly, cabin1 and calcineurin were shown to co-localise in the brain (Lai *et al*, 1998). It has been reported cabin1/cain inhibits both T-cell activation by downregulation of calcineurin phosphatase activity and consequent attenuates NFAT activation (Sun *et al*, 1998) and synaptic vesicle endocytosis (Lai *et al*, 2000).

1.2.7c A-Kinase-Anchoring Protein (AKAP79)

AKAP79 is localised in T-cells and neurones (Kashishian *et al*, 1998) and has been classed as a scaffolding protein that can anchor multiple proteins in a complex with the potential of targeting both kinases and phosphatases in specific sub-cellular sections to regulate protein and receptor activity (Dell'Acqua *et al*, 2002). Specifically, AKAP79 has been shown to interact with Cn A in COS cells and act as an inhibitor of calcineurin activity as over-expression of AKAP79 in HEK293T cells results in a 95% down-regulation of NFAT activation compared to the control in response to PMA and ionophore stimulation (Kashishian *et al*, 1998). In addition, co-expression of calcineurin and AKAP79 in COS-7 cells resulted in translocation of calcineurin from the cytoplasm to the membrane (Dell'Acqua *et al*, 2002). Therefore it is proposed the down-regulation of NFAT activity observed is a result of the interaction between calcineurin and AKAP79 which maintains calcineurin at the membrane out of spatial contact with NFAT (Kashishian *et al*, 1998).

1.2.7d Calcineurin Homologous Protein (CHP)

The calcineurin homologous protein (CHP) is a calcium binding protein that has a high percentage of homology of 65% and 59% to Cn B and CaM respectively.

Over-expression of CHP in Jurkat T-cells results in inhibition of PMA and ionophore-induced NFAT activity. Interestingly, ectopic expression of CHP in HeLa cells prevented nuclear localisation of NFAT in response to PMA and ionophore stimulation. Furthermore, CHP was shown to inhibit calcineurin phosphatase activity and interact with Cn A. It was therefore proposed CHP disrupts the interaction between calcineurin A, calcineurin B and calmodulin which consequently attenuates the calcineurin/NFAT signalling pathway (Lin *et al*, 1999).

1.2.7e Regulator of Calcineurin 1 (RCAN1)

The regulator of calcineurin 1 (RCAN1), also referred to as Down syndrome critical region 1 (DSCR1), myocyte-enriched calcineurin interacting protein 1 (MCIP1), calcipressin 1 and Adapt 78, is composed of 7 exons. The first 4 exons are alternative with the two main isoforms RCAN1.1 and RCAN1.4 being generated with the inclusion of exon 1 or exon 4 respectively (Holmes *et al*, 2010). Isoforms are produced via alternative promoter usage. RCAN1.4 contains 15 NFAT binding sites upstream of exon 4 and its expression is activated in response to calcineurin/NFAT signalling whereas RCAN1.1 expression is not (Yang *et al*, 2000; Yao and Duh, 2004; Holmes *et al*, 2010).

RCAN1 was shown to interact with calcineurin and this has been mapped to the region encompassing nucleotides 338-352 of calcineurin which is located between the Cn A catalytic domain and the Cn B binding region (Rothermel *et al*, 2000; Fuentes *et al*, 2000). RCAN1 was shown to inhibit calcineurin activity as seen by a reduction in calcineurin dependent gene transcription or attenuation of calcineurin/NFAT dependent promoter activity (including its own)

due to prevention of NFAT nuclear localisation (Rothermel *et al*, 2000; Fuentes *et al*, 2000; Yao and Duh, 2004; Hesser *et al*, 2004). This downregulation of calcineurin activity was shown to be dependent on the interaction between the two proteins (Fuentes *et al*, 2000). It is therefore suggested RCAN1.4 acts as negative feedback inhibitor of calcineurin activity (Yao and Duh, 2004).

1.2.7f Plasma Membrane Calcium ATPase (PMCA)

PMCA has been shown to interact with calcineurin in a variety of cells including endothelial cells (Holton *et al*, 2010a), cardiomyocytes (Wu *et al*, 2009), PC12 cells (Kosiorek *et al*, 2011), HEK293 cells (Buch *et al*, 2005) and breast cancer cells (Holton *et al*, 2007). Transfection of HEK293 cells with a plasmid encoding PMCA4b has been shown to downregulate NFAT activation by 60% compared to the control in response to PMA and calcium ionophore (A23187) stimulation, showing PMCA4 is an inhibitor of the calcineurin/NFAT pathway (Buch *et al*, 2005). A similar result was observed for the PMCA2b isoform (Holton *et al*, 2007).

Given the adverse effects associated with the high concentration requirement of calcineurin inhibitors such as CsA in clinic (Crabtree, 1999; Matrínez-Martínez and Redondo, 2004), research has focused on the identification of endogenous down-regulators of the calcineurin/NFAT pathway as future therapeutic targets. Therefore in this thesis we have focused on the plasma membrane calcium ATPase 4 (PMCA4) as an endogenous inhibitor of calcineurin-dependent angigoensis.

1.3 Plasma Membrane Calcium ATPases (PMCAs)

Plasma membrane calcium ATPases (PMCAs) are membrane proteins involved in calcium extrusion from the cytoplasm to the external environment. They are a member of the P-type family of ATPases defined by their ability to become phosphorylated during the transportation of calcium (Carafoli and Brini, 2000). PMCAs have an approximate MW of 138 kDa and in erythrocytes constitute no more than 0.05% of the total membrane protein (Verma *et al*, 1988).

1.3.1 Mechanism of Calcium Removal

PMCAs exist in two main conformations known as E1 and E2. In the E1 state the calcium (Ca^{2+}) binding site faces the cytosol and has a high affinity for calcium. A conserved aspartic acid residue within the PMCA structure (Aspartate 672 in human PMCA4) becomes phosphorylated with concomitant hydrolysis of an ATP molecule and PMCA converts from the E1 to the E2 conformation resulting in the exposure of Ca^{2+} to the extracellular space. In this state Ca^{2+} has a lower affinity for the binding site and is released. Dephosphorylation of the Aspartate residue returns the pump to the E1 conformation ready for the cycle to begin again (Carafoli and Brini, 2000). One calcium ion is transported for one ATP molecule hydrolysed and results in the intake of a hydrogen ion (H^+) (Di Leva *et al*, 2008).

1.3.2 Structure of PMCAs

The putative structure of PMCAs consists of ten transmembrane (TM) domains with the majority of the protein being cytosolic. The cytosolic portion of the pump is further subdivided into four main units: The N-terminus and the first intracellular loop between TM2 and TM3 which contains the binding site for activatory acidic phospholipids and a splice site (site A) (the transduction domain), a large catalytic intracellular loop between TM4 and TM5 which contains the ATP binding site and the Aspartate that becomes phosphorylated in the calcium removal cycle (the phosphorylation domain), and the C-terminus that contains another site for splicing (site C) and a binding domain for calmodulin that is involved in auto-inhibition of the pump (the nucleotide binding domain) (Fig. 1.3.1) (Strehler and Zacharias, 2001; Di Leva *et al*, 2008).

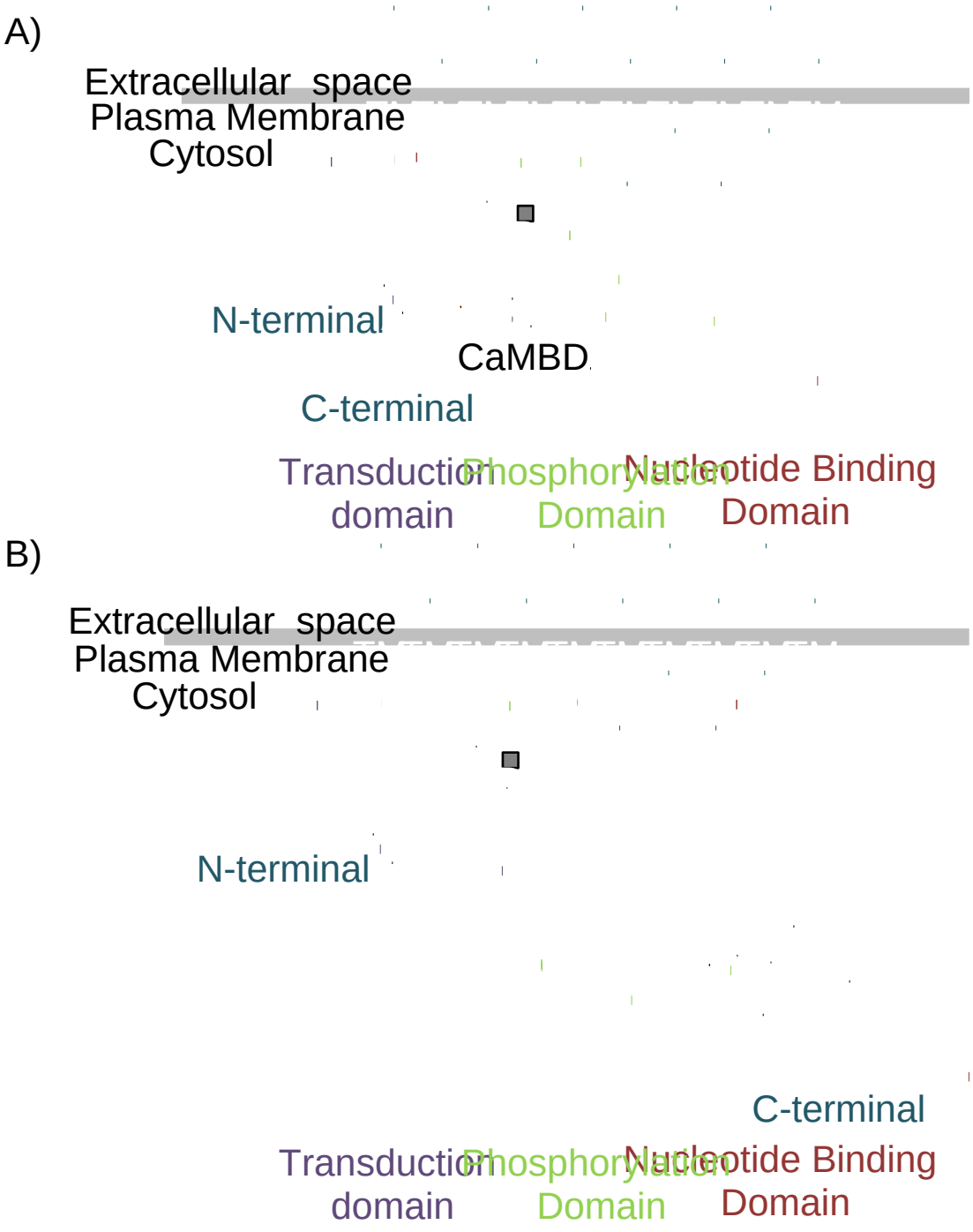

A)
Extracellular space
Plasma Membrane
Cytosol
N-terminal
CaMBD
C-terminal
Transduction domain
Phosphorylation Domain
Nucleotide Binding Domain
B)
Extracellular space
Plasma Membrane
Cytosol
N-terminal
C-terminal
Transduction domain
Phosphorylation Domain
Nucleotide Binding Domain

Fig. 1.3.1 The putative structure of PMCAs (adapted from Di Leva *et al*, 2008; Strehler and Zacharias, 2001). PMCAs contain 10 transmembrane domains (TM) with three main cytosolic domains: The transduction domain containing the N-terminus and the first intracellular loop between TM2 and TM3 which includes both splice site A (A) and a phospholipid binding domain (PL BD). The phosphorylation domain includes the large cytosolic loop between TM4 and TM5 which contains an Aspartate residue (conserved in all isoforms) that becomes phosphorylated during the reaction cycle (D). The nucleotide binding domain includes the C-terminal tail which contains a calmodulin binding domain (CaMBD) and splice site C (C). A) When the calmodulin binding domain interacts with two sites of the PMCA pump, one located in the first intracellular loop and one in the cytosolic loop between TM4 and TM5, the pump is in an inhibited state. B) When calmodulin (CaM) binds to the CaMBD it removes the interaction of the auto-inhibitory site with the two pump receptor sites and consequently removes the auto-inhibition, stimulating pump activity.

1.3.3 PMCA Isoforms

There are four main PMCA isoforms: PMCA1, PMCA2, PMCA3 and PMCA4 encoded by four independent genes *ATP2B1*, *ATP2B2*, *ATP2B3* and *ATP2B4* respectively (Di Leva *et al*, 2008). RNA alternative splicing at sites A and C generates further isoform diversity (Strehler and Zacharias, 2001). To date more than 20 varieties of PMCAs have been reported.

1.3.3a Splice Site A

At splice site A, different isoforms (denoted w,x,y and z) are generated by the inclusion or exclusion of up to 3 exons. The ‘w’ variant contains all three additional exons and variant ‘z’ includes no exons (Fig. 1.3.2). For all the PMCA isoforms insertion of an exon at this site does not affect the reading frame (Strehler and Zacharias, 2001). It has been reported that splicing at site A regulates PMCA targeting to specific membranes (Chicka and Strehler, 2003).

1.3.3b Splice Site C

Isoforms generated by alternative splicing at site C are labelled a,b,c,d,e and f, where variant ‘a’ contains one exon and variant ‘b’ has no inserted exons (Fig. 1.3.2). Inclusion of exons at this splice site in isoform ‘a’ pumps results in a shift in the reading frame leading to the truncation of the C-terminal region of the protein. PMCAb variants maintain fully functioning C-terminal regions. Splice site C is found within the calmodulin binding region (Fig 1.3.1) thus splicing at this site affects the affinity of the pump for calmodulin (Strehler and Zacharias,

2001). For example, PMCA4b isoforms have a higher affinity for calcium and calmodulin then PMCAa isoforms (Enyedi *et al*, 1994).

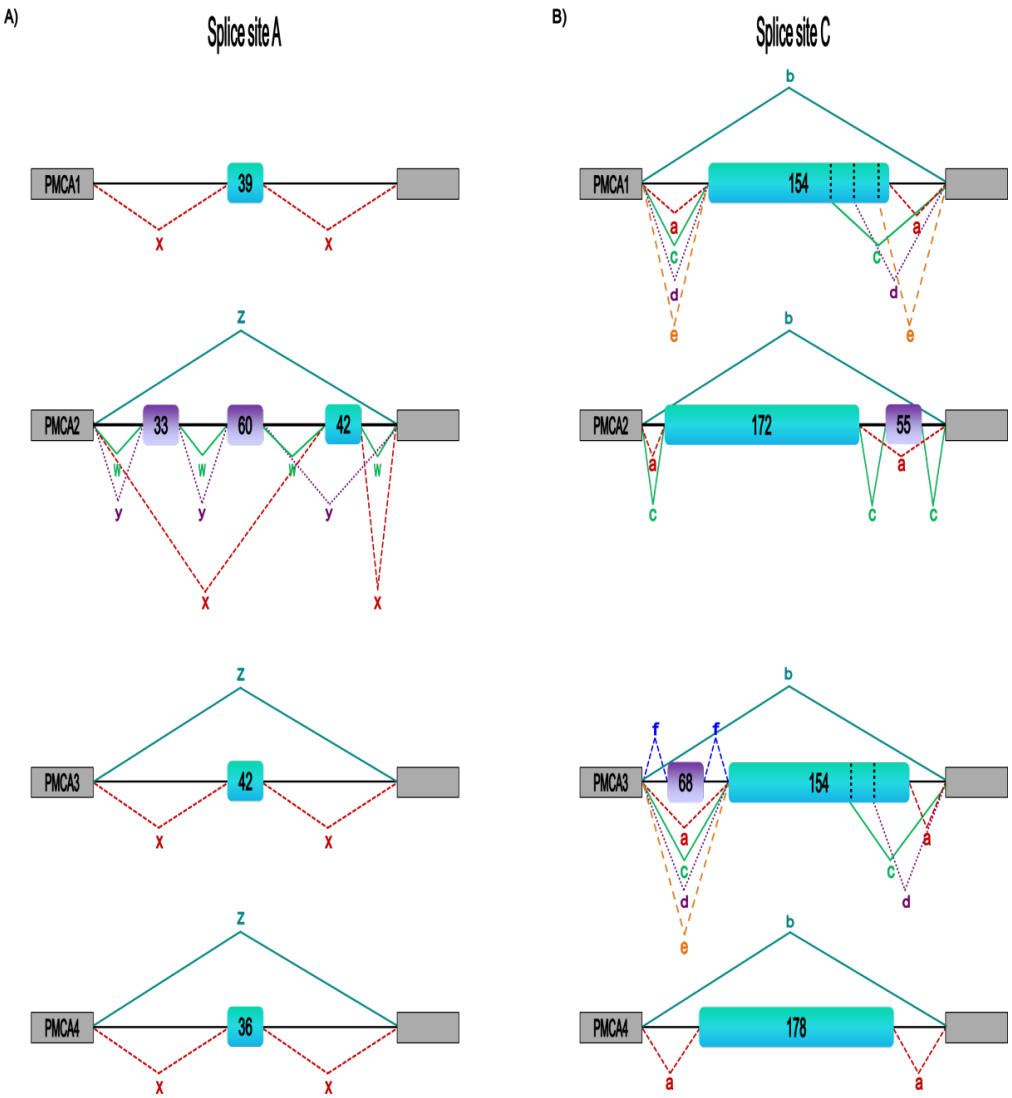


Fig. 1.3.2 Generation of isoform diversity by alternative splicing at splice site A and C (adapted from Strehler and Zacharias, 2001). Images not drawn to scale. Blue bars represent inserted exons that are equivalent among the isoforms, the number inside denotes the size in nucleotides. Purple boxes represent additional exons not occurring in all isoforms. A) Diagram of potential isoforms at splice site A. All isoforms denoted ‘z’ contain no inserted exon, isoforms ‘x’ result from the insertion of one exon. Only PMCA2 has additional isoforms due to the insertion of two more exons

at this site. B) Isoforms occurring from splicing at site C. All isoforms denoted ‘b’ contain no exons, one exon is inserted in ‘a’ isoforms. A greater number of splice variants occurs at site C as there are further splice sites within exons (represented by dashed line on the exon itself) and additional exons are included in both PMCA2 and PMCA3 isoforms.

1.3.3c Expression of PMCA Isoforms

PMCA1 and PMCA4 are found throughout most tissues and have a wide distribution in adults. In comparison PMCA2 and 3 display a more specific cellular and tissue distribution. PMCA2 is localised to the brain, cochlear hair cells of the inner ear, lactating mammary gland, uterus, kidney and liver. PMCA3 is predominantly found in the brain (Strehler and Zacharias, 2001).

1.3.4 PMCA Knockouts

PMCA knockout (-/-) mice have been generated to identify the physiological function of PMCA isoforms *in vivo* (Prasad *et al*, 2004).

1.3.4a PMCA1

PMCA1$^{-/-}$ mice do not survive past embryonic development suggesting a ‘housekeeping’ role for PMCA1 (Okunade *et al*, 2004). Recently, Kobayashi *et al*, 2012 generated a specific knockout of the PMCA1 gene *ATP2B1* in vascular smooth muscle cells of mice that showed high blood pressure levels. This phenotype was associated with an increase in both intracellular calcium levels and femoral artery vasoconstriction in response to phenylephrine (PE) stimulation. This data highlights a further role for PMCA1 specifically in the regulation of blood pressure (Kobayashi *et al*, 2012).

1.3.4b PMCA2

The isoform PMCA2w/a is highly expressed within the plasma membrane of the stereocilia of inner ear hair cells (Carafoli, 2011), highlighting the pivotal role of PMCA2 in the auditory system as PMCA2$^{-/-}$ mice are profoundly deaf (Kozel *et al*, 1998). Moreover, ablation of PMCA2 also results in balance impediments that are proposed to be linked to the loss of otoconia in the knockout animals (Kozel *et al*, 1998).

PMCA2b is the predominant isoform expressed in the apical membrane of mammary gland cells and its protein expression increases dramatically during lactation (up to 100 fold increase) (Reinhardt *et al*, 2000). PMCA2$^{-/-}$ mice have a significantly reduced calcium content in their milk (up to 60% less compared to wild-type animals) (Reinhardt *et al*, 2004) suggesting that PMCA2 acts as a macro-calcium transporter to enrich milk with calcium during lactation (Reinhardt *et al*, 2000).

PMCA2 is also involved in the regulation of apoptosis in the physiological process of mammary gland involution that occurs upon lactation cessation. A study using PMCA2 deafwaddler-2J knockout mice, demonstrated that loss of PMCA2 on day 18 of pregnancy resulted in an increase in apoptosis of mammary epithelial cells. Likewise, ionomycin-induced apoptosis in mammary epithelial cells from PMCA2$^{-/-}$ mice is significantly higher than that of wild-type cells. These results suggest an apoptosis-protective function for PMCA2 (VanHouten *et al*, 2010).

1.3.4c PMCA3

A knockout model of PMCA3 has not been created to date.

1.3.4d PMCA4

PMCA4$^{-/-}$ male mice have been reported to be infertile (Schuh *et al*, 2004) due to a reduction in the motility and directed movement of sperm (Schuh *et al*, 2004; Okunade *et al*, 2004).

PMCA4 has been shown to have a role in the heart as PMCA4$^{-/-}$ results in an increase in cardiac hypertrophy (enlargement of the heart) in response to pressure overload by transverse aortic constriction (TAC) compared to the wild-type. An increase in myocyte surface area and pulmonary edema were also observed. Interestingly, hypertrophy as a result of exercise was not altered between PMCA4$^{-/-}$ and wild-type mice highlighting a specific role for PMCA4 as a down-regulator of pathological hypertrophy (Wu *et al*, 2009). PMCA4$^{-/-}$ mice also have an increase in cardiac contraction *in vivo* which was proposed to be a result of a decrease in membrane localised nNOS due to loss of PMCA4 which alters microdomain signalling, leading to a decrease in cGMP and phosphodiesterase 2 (PDE2) activity, an increase in cAMP and consequently phosphorylation of ryanodine receptors and activation of L-type calcium channels which increases contraction. Interestingly, the loss of PMCA4 had no effect on the rate of overall calcium removal (Mohamed *et al*, 2011).

PMCA4 has also been implicated in the regulation of osteoclasts and consequently bone formation. *In vivo* studies using PMCA1$^{+/-}$ or PMCA4$^{-/-}$ mice

have shown that femurs from 6 week old mice have a lower bone volume and decreased mineralization and density compared to wild-type mice (Kim *et al*, 2012). Study of trabecular bone sections and osteoclasts derived from PMCA1$^{+/-}$ or PMCA4$^{-/-}$ mice confirmed apoptosis was increased with the loss of PMCA1or 4 protein suggesting that PMCA1 and 4 also share the apoptosis protective function observed for PMCA2 (Kim *et al*, 2012).

PMCA4 has been hypothesised to play a specific role in bladder smooth muscle contraction by regulation of the acetycholine signalling pathway as PMCA4$^{-/-}$ in mice bladder smooth muscle cells results in reduced contraction after carbachol stimulation, however there was no significant difference in general calcium clearance compared to the wild-type which is in contrast to that found for PMCA1$^{+/-}$ (Liu *et al*, 2007).

1.3.5 Regulation of PMCA Activity

PMCA activity is regulated by a variety of mechanisms including modification by kinases, interaction with proteins, cleavage and self-association which result in an alteration of PMCA activity (Di Leva *et al*, 2008).

1.3.5a Calmodulin

The calmodulin binding domain (CaMBD) interacts with two sites of the PMCA pump; one located in the intracellular loop between TM2 and 3 and another in the catalytic loop between TM4 and TM5 (Falchetto *et al*, 1991; Falchetto *et al*, 1992) (Fig. 1.3.1). Calmodulin increases the affinity of the ATPase pump for calcium and also its activity (Niggli *et al*, 1981). It is proposed that binding of calmodulin to the CaMBD displaces its interactions with the pump consequently removing the auto-inhibition (Di Leva *et al*, 2008). The concept of the CaMBD acting as an auto-inhibitory mechanism was confirmed with a mutant PMCA4b

pump that lacks the last 120 C-terminal amino acids (PMCA4b (ct120)) and therefore no longer contains a CaMBD. PMCA4b (ct120) was shown to be constitutively active, calmodulin independent and had 10 times greater activity than the wild-type (Enyedi *et al*, 1993).

1.3.5b Acidic Phospholipids

Binding sites for acidic phospholipids have been located to the intracellular loop between TM2 and TM3 and also the CaMBD within the PMCA structure (Di Leva *et al*, 2008). Specifically, the acidic phospholipids; phosphatidylserine and phosphatidic acid both increase, up to 4 times, the activity of PMCAs isolated from human erythrocytes. Stimulation of the pump with both calmodulin and phospholipid is additive and is more pronounced at low concentrations of phospholipid suggesting they share a binding site (Brodin *et al*, 1992).

1.3.5c Oligomerization

Fluorescence resonance energy transfer (FRET) analysis of PMCAs isolated from erythrocytes labelled with either a donor or acceptor fluorophore, showed that PMCAs associate and can therefore form oligomers, with the pump activity being enhanced by oligomerization (Kosk-Kosicka *et al*, 1989). It was later established that oligomerization of PMCAs occurs via its calmodulin binding domain (Vorherr *et al*, 1991).

1.3.5d Phosphorylation

PMCAs are phosphorylated by a variety of kinases at various residues within its structure (Di Leva *et al*, 2008).

Cyclic AMP (CAMP)-dependent protein kinase, protein kinase A (PKA), directly phosphorylates PMCAs from both sarcolemmal and erythrocyte membranes which consequently increases ATPase activity (Neyses *et al*, 1985).

Protein kinase C (PKC) also activates the calcium transport activity of PMCAs by phosphorylating a threonine residue located in the CaMBD and thus preventing the auto-inhibitory conformation of the pump (Wang *et al*, 1991; Hofmann *et al*, 1994).

In some cases PMCA phosphorylation inhibits the function of the pump. For example, phosphorylation of tyrosine 1176 of PMCA4b by activated $pp60^{src}$ kinase turns off the activity of the pump by up to 75% (Dean *et al*, 1997).

1.3.5e Calpain Cleavage

Calpain is a calcium dependent endopeptidase known to act on a number of proteins. It has been reported to digest the C-terminal region of PMCAs. This cleavage results in the formation of an approximately 124 kDa version of the pump that lacks the auto-inhibitory domain and is constitutively active (James *et al*, 1989).

1.3.6 PMCA Membrane Localisation in Relation to Involvement in Signalling Pathways

1.3.6a Lipid Rafts

Lipid rafts are specialised membrane domains characterised by high levels of cholesterol and sphingolipids. Rafts cluster together bringing various molecules into close proximity and allow the possibility of modification of these proteins which has the potential to initiate signalling pathways (Simons and Toomre, 2000). In pig cerebellum synaptic plasma membrane, PMCAs localise to lipid rafts in an isoform dependent manner. PMCA4 is found within the lipid raft density fraction whereas PMCA1, 2 and 3 are not (Sepúlveda *et al*, 2006).

1.3.6b Caveolae

Caveolae are membrane invaginations that are implicated in the regulation of cellular signalling pathways (Simons and Toomre, 2000). PMCAs are reported to localise to caveolae in plasma membranes in a variety of cells, including endothelial cells, suggesting that targeted localisation of PMCA proteins may lead to more significant changes in calcium concentration in defined regions of the cytosol (Fujimoto, 1993). PMCA4b but not PMCA4a localisation to caveolae is dependent on caveolin-1, a protein essential for correct caveolae formation. In bovine tracheal smooth muscle, disruption of caveolae formation also decreases the calcium extrusion properties of PMCA4, suggesting caveolae are required for the correct functioning of PMCA (El-Yazbi *et al*, 2008).

1.3.7 PMCA Interacting Partner Proteins

A variety of cytoplasmic cellular proteins have been demonstrated to interact with PMCAs. These interactions occur at various positions of the PMCA structure (Fig. 1.3.3) and have a variety of effects on the activity of both PMCA and the partner protein (Di Leva *et al*, 2008) which consequently regulates their corresponding signalling pathways. This is in agreement with the localisation of PMCAs in membrane micro-domains specialised in signal transduction such as caveolae (Holton *et al*, 2010b). Whether the interaction with these proteins is dependent on caveolae localisation is not known at present.

1.3.7a Protein Interaction With the N-Terminal Region of PMCA

The N-terminal domain of the PMCA pumps is the most variable portion of the protein between the four isoforms, with roughly 50% amino acid similarity. Only one partner protein, the small acidic protein 14-3-3ϵ, has been identified to date to interact with this region (Fig. 1.3.3) (Rimessi *et al*, 2005). There are several

known isoforms of the small acidic protein 14-3-3 including β, η, ζ, σ, θ, ϵ and γ that have been implicated in the regulation of cellular signalling pathways, localisation of molecules and processes such as apoptosis (Linde *et al*, 2008). PMCAs interact with 14-3-3ϵ in an isoform dependent manner. PMCA1, 3 and 4 have been shown to interact with 14-3-3ϵ whereas PMCA2 does not (Rimessi *et al*, 2005; Linde *et al*, 2008). Studies on the functional consequences of the interaction PMCA/14-3-3ϵ have shown that co-expression of the two proteins leads to inhibition of the calcium extrusion properties of PMCAs highlighting 14-3-3ε as a down-regulator of PMCA pump activity (Linde *et al*, 2008).

1.3.7b Protein Interaction With the Catalytic Core of PMCA

Unlike the N-terminal region of PMCA pumps, the large intracellular loop between TM4 and TM5 involved in calcium removal has high similarity in its amino acid sequence (up to 80%) among the isoforms (Buch *et al*, 2005). A number of interactions with partner proteins have been mapped to this domain of PMCAs (Fig. 1.3.3) (Di Leva *et al*, 2008).

1.3.7b(i) Ras-Associated Factor 1 (RASSF1)

RASSF1 is a tumour suppressor protein that is implicated in Ras signalling pathways, in particular Ras-mediated apoptosis (Vos *et al*, 2000). The Ras signalling pathway also regulates Erk activity and consequently cell proliferation (Force and Bonventre, 1998). RASSF1 proteins are expressed ubiquitously and have multiple splice variants with the major isoforms RASSF1A and 1C, which have both been reported to interact with PMCA4b in HEK293 cells, specifically via amino acids 652-748 of PMCA4b (Armesilla *et al*, 2004). Additionally, in rat neonatal cardiomyocytes both endogenous PMCA4 and RASSF1 proteins co-

localise to the membrane further confirming the PMCA4/RASSF1A interaction. The interaction of the two proteins results in down-regulation of the epidermal growth factor (EGF)-mediated activation of Erk, suggesting that PMCA acts as a negative regulator of Ras-mediated signalling pathways via an interaction dependent mechanism with RASSF1 (Armesilla *et al*, 2004).

1.3.7b(ii) Endothelial Nitric Oxide Synthase (eNOS/NOS III)

eNOS is a calcium/calmodulin dependent enzyme that catalyses the production of NO from L-arginine and molecular oxygen (Förstermann and Sessa, 2012). eNOS plays a fundamental role in the regulation of critical vascular responses including dilation of blood vessels, protection from atherosclerosis and regulation of blood pressure (Förstermann and Sessa, 2012). Endogenous PMCA1, 2 and 4 have been demonstrated to interact with eNOS in human endothelial cells (Holton *et al*, 2010a). This interaction occurs via the large catalytic loop of PMCAs. The PMCA/eNOS interaction results in an increase in the inhibitory phosphorylation of the eNOS residue Thr-495. Functionally, ectopic expression of PMCA2b or 4b in endothelial cells both resulted in a reduction in the production of NO in response to acetylcholine stimulation or ionophore (Holton *et al*, 2010a).

1.3.7b(iii) Calcineurin

The first indication of an interaction between PMCA and calcineurin was reported by Buch *et al*, 2005 between ectopically expressed PMCA4b and calcineurin in HEK293 cells. Functionally, over-expression of PMCA4b resulted in up to 60% reduction in the activity of the calcineurin/NFAT pathway identifying a new role for PMCA4b as a negative regulator of this pathway (Buch

et al, 2005). Additionally, PMCAs have been found to interact with calcineurin in an isoform-dependent manner in breast cancer MCF-7 cells, with calcineurin binding predominantly to PMCA2, moderately to PMCA4 and no interaction occuring with PMCA1 (Holton *et al*, 2007). Interestingly, our group has recently reported that disruption of the interaction PMCA2/calcineurin in a panel of breast cancer cell lines increased calcineurin-mediated apoptosis and sensitised the breast cancer cells to paclitaxel-induced cytotoxicity underscoring the importance of the interaction PMCA2/calcineurin in breast cancer progression (Baggott *et al*, 2012). Additionally, in the cardiovascular system endogenous PMCA1, 2 and 4 have also been shown to interact with calcineurin in endothelial cells (Holton *et al*, 2010a) and in cardiomycocytes the interaction PMCA4/calcineurin has been shown to play a critical role as a negative regulator of pathological cardiac hypertrophy (Wu *et al*, 2009). Finally in PC12 neuronal cells PMCAs interaction with calcineurin is isoform dependent and is proposed to be a regulator of dopamine secretion by these cells (Kosiorek *et al*, 2011).

1.3.7b(iv) Syntrophin

Mouse α-1 syntrophin has been shown to interact with the large catalytic intracellular loop of human PMCA1b and 4b in HEK293 cells and PMCA, syntrophin and nNOS have been reported to form a macromolecular complex (Williams *et al*, 2006). The interaction PMCA/syntrophin has been proven to act synergistically to inhibit nNOS activity (upto 80% inhibition) (Williams *et al*, 2006).

1.3.7c Protein Interaction With the C-Terminal Domain of PMCA

Interactions with the C-terminal region of PMCA occur via a Post synaptic density protein, Drosophila disc large tumour suppressor and Zonula occludens-1 protein (PDZ) binding domain. Protein interactions involving the PDZ domain require the minimum sequence E-S/T-X-V (where X represents any amino acid) (DeMarco and Strehler, 2001). PMCAb isoforms contain a PDZ binding domain (either ETSL or ETSV for PMCA1/2/3 and PMCA4 respectively) at the C-terminus allowing their interaction with various PDZ containing partner proteins (Fig. 1.3.3) (Kim *et al*, 1998).

1.3.7c(i) Membrane Associated Guanylate Kinase (MAGUK)

MAGUKs have a role in the regulation of cellular signalling specifically at the membrane (DeMarco and Strehler, 2001) and contain three PDZ domains (Kim *et al*, 1998). One member of this family is the synapse associated protein (SAP) which is proposed to act as a scaffolding protein at the membrane. There are multiple SAPs including SAP90/ post-synaptic density protein of 95 kDa (PSD-95), SAP93/Chapsyn-110, SAP97/hDlg and SAP102/NE-Dlg (DeMarco and Strehler, 2001). PMCA2b and 4b have been shown to interact with a variety of SAPs such as SAP90, SAP93 and SAP97, with SAP102 specifically interacting with only PMCA4b (DeMarco and Strehler, 2001; Kim *et al*, 1998). Interstingly the PMCA ‘a’ isoforms fail to interact with SAP97 showing an isoform selective interaction with SAPs (Kim *et al*, 1998). In epithelial Madin-Darby Canine Kidney (MDCK) cells PMCA4b and SAP97 co-localise to the basolateral membrane. It is hypothesised interaction of PMCAs with SAPs may localise the pump within signalling complexes at specific sites of the membrane (DeMarco and Strehler, 2001).

1.3.7c(ii) Calcium /Calmodulin Dependent Serine Protein Kinase (CASK)

CASK is also classed as a MAGUK family member and contains a PDZ domain. Additionally, it contains a guanylate kinase-like domain allowing its interaction with Tbr-1, a T-box transcription factor. CASK and Tbr-1 co-localise to the nucleus where they induce the transcription of promoters containing T-elements (Schuh *et al*, 2003b). Co-immunoprecipitation experiments with both rat brain and kidney lysates demonstrated the interaction between endogenous PMCA and CASK which results in a significant inhibition of T-element driven reporter vectors, indicating that PMCA4b down-regulates CASK activity (Schuh *et al*, 2003b).

1.3.7c(iii) Na^+/H^+ Exchanger Regulatory Factor 2 (NHERF2)

There are four members within the NHERF family NHERF1-4. In particular NHERF2 is known to act as a scaffold of molecular complexes due to its two PDZ domains and also an ezrin-radixin-moesin binding domain, allowing its interaction with ion channels or receptors and the actin cytoskeleton respectively (Kruger *et al*, 2009). NHERF2 has been found to interact with PMCA in an isoform dependent manner associating with PMCA2b but not 4b (DeMarco *et al*, 2002). As PMCA1 and 3 have the same terminal sequence as PMCA2b it is hypothesised they will also interact with NHERF2. PMCA2b and NHERF2 were shown to co-localise to the apical membrane in MDCK cells

(DeMarco *et al*, 2002). Silencing of NHERF2 results in a decrease in the rate of calcium recovery as there is a reduction in calcium removal by PMCA. This was suggested to be the result of the reduction in PMCA localisation to the plasma membrane due to the loss of NHERF2 (Kruger *et al*, 2009).

1.3.7c(iv) CLP36

Human CLP36 is a 38 kDa protein that contains a PDZ domain within its N-terminus. CLP36 also forms a complex with α-actinin-1 and as a result interacts with actin filaments and therefore the cytoskeleton in both endothelial cells and activated platelets (Bauer *et al*, 2000). CLP36 has also been shown to interact with PMCA in resting platelets and were reported to be in a complex with α-actinin and actin that upon activation of platelets becomes associated with the cytoskeleton. It is therefore proposed that PMCAs interaction with CLP36 acts to mediate its association with the cytoskeleton (Bozulic *et al*, 2007).

1.3.7c(v) Neuronal Nitric Oxide Synthase (nNOS/NOS-I)

nNOS is another nitric oxide synthase enzyme that is calcium/calmodulin dependent (Förstermann and Sessa, 2012). Immunoprecipitation and pull-down assays identified the interaction of ectopic PMCA4b and nNOS in HEK293 cells via a PDZ domain (Schuh *et al*, 2001). nNOS activity, measured by cGMP production, was inhibited in a dose dependent manner in relation to increasing PMCA4b expression in HEK293 cells, indicating the functional significance of the interaction between the two proteins (Schuh *et al*, 2001). It is hypothesised PMCAs down- regulation of nNOS activity is due to the calcium extrusion activity of the pump. In agreement with this theory a mutant form of PMCA4b at residue Asp672 (which significantly reduces calcium removal by the pump)

failed to inhibit nNOS activity to the same extent as the wild-type (Schuh *et al*, 2001). Additionally, a PMCA pump with the last 120 residues removed (resulting in a constitutively active pump with no PDZ binding domain) has no effect on nNOS activity compared to the control (Schuh *et al*, 2001).

PMCA1 and 4 have also been shown to interact with nNOS in cardiac cells (Williams *et al*, 2006). In the heart, PMCA4 has been proposed to act as a scaffold to regulate nNOS activity. The PMCA/nNOS interaction down-regulates the β-adrenergic contractile response as the mutant pump, PMCA4ct120 which has the PDZ domain removed and therefore cannot interact with nNOS, had no effect on β-adrenergic contraction. Interestingly, over-expression of PMCA4b had no effect on general excitation-contraction coupling as the rate of calcium removal was unchanged from the wild-type (Oceandy *et al*, 2007).

Furthermore, Schuh *et al*, 2003a, demonstrated that mice over-expressing PMCA4b in vascular smooth muscle cells specifically, had an increase in blood pressure *in vivo*. It was hypothesised this result occurs due to PMCAs interaction with and consequently downregulation of nNOS activity which would regulate vascular tone. However, over-expression of PMCA4b had no impact on endothelium dependent relaxation. PMCA4 was therefore suggested to have a role not in general contraction, but in signalling pathways which regulate contraction, in this case via nNOS (Schuh *et al*, 2003a).

1.3.7c(vi) PMCA Interacting Single PDZ Protein (PISP)

PISP is a single PDZ containing protein. All PMCAb splice variants interact with PISP (Goellner *et al*, 2003). PISP does not interact with a truncated mutant of PMCA4b missing six amino acids at the C-terminus suggesting that the

interaction between the two proteins is via the PDZ binding region (Goellner *et al*, 2003). At present, it is not clear what the functional consequences of the interaction PMCA/PISP are. In reconstituted human erythrocyte membranes PISP has no effect on PMCA pump activity (Goellner *et al*, 2003). It is hypothesised that PISP may act as a transporter protein for PMCA localisation to the membrane, although this has yet to be determined experimentally (Goellner *et al*, 2003).

1.3.7c(vii) Ania-3

Ania-3 is a member of the Homer protein family. Homer proteins are involved in the regulation of the interaction of metabotropic glutamate receptors (mGluR) with other receptors such as IP_3 which could potentially regulate calcium concentration within the cytosol. Ania-3, like all Homer proteins, contains a PDZ sequence (Sgambato-Faure *et al*, 2006). In COS-7 cells Ania-3 interacts with all b splice variants of PMCA1-4. In MDCK epithelial cells PMCA2b and Ania-3 have been reported to co-localise to the plasma membrane and in rat hippocampal neurons the two proteins co-localise in the soma and dendrites. The C-terminal region of PMCA was found to be essential for the interaction between the two proteins (Sgambato-Faure *et al*, 2006). More recently it has been shown in rat hippocampal neurones knockdown of the Homer 1 protein results in a reduction in the calcium removal by PMCAs (Salm and Thayer, 2012). It is hypothesised Homer 1 proteins mediate their effect on PMCA activity by interacting with the C-terminal region and preventing the association of the auto-inhibitory domain with its site in the PMCA pump (Salm and Thayer, 2012).

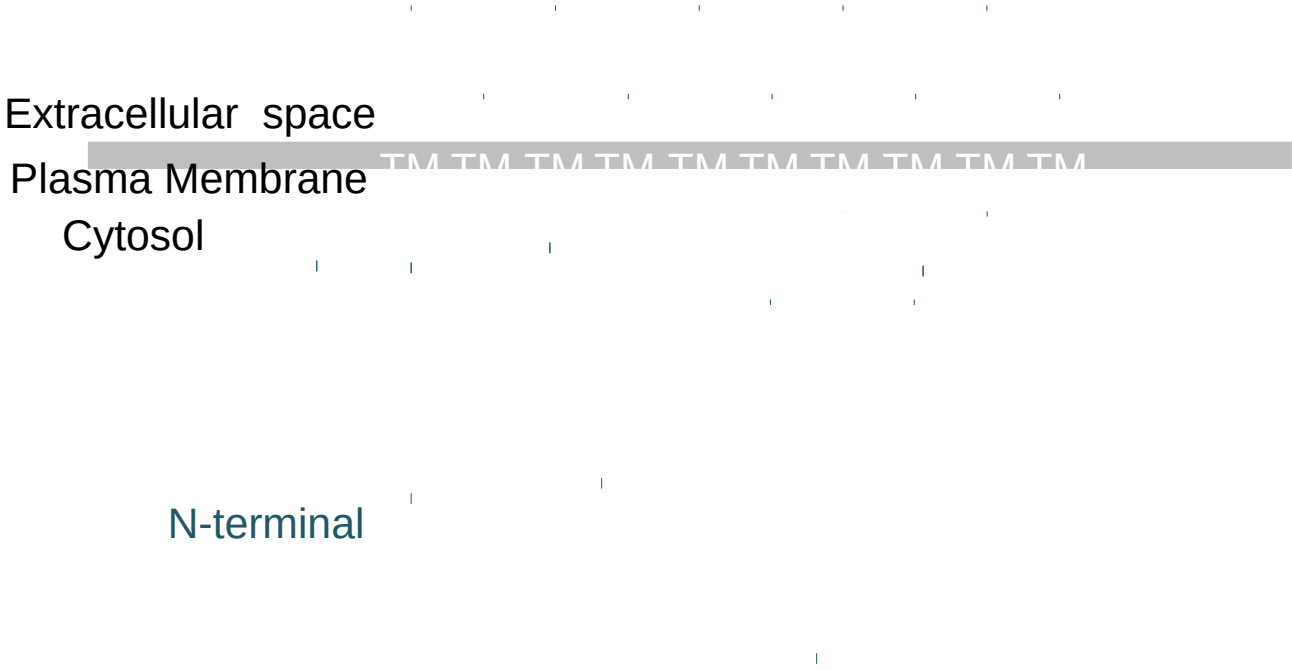


Fig. 1.3.3 PMCA and its partner proteins (adapted from Di Leva *et al*, 2008). Interactions with partner proteins occur at three main domains of PMCA: the N-terminal, the large cytosolic loop between transmembrane (TM) domain 4 and 5 and the C-terminal tail containing a PDZ-binding domain. Only one protein to date has been reported to interact with the N-terminal domain which is the 14-3-3ϵ protein. Endothelial nitric oxide synthase (eNOS), calcineurin A, ras-associated factor 1(RASSF1) and syntrophin bind to the second, large intracellular loop between TM4 and TM5. Syntrophin has been proposed to form a macromolecular complex with neuronal nitric oxide synthase (nNOS) which binds to the c-terminal region of PMCA via a PDZ motif. Many other PDZ-containing partner proteins such as PMCA interacting single PDZ protein (PISP), membrane associated guanylate kinase (MAGUK), Na^+/H^+ exchanger regulatory factor 2 (NHERF2), CLP36, Ania-3 and

calcium /calmodulin dependent serine protein kinase (CASK) bind to the PDZ-binding region present in the C-terminal end of PMCAb isoforms.

1.4 Aims and Hypothesis

It has previously been reported PMCA4 interacts with calcineurin and negatively-regulates the calcineurin/NFAT signalling pathway in HEK293 mammalian cells and cardiomyocytes (Buch *et al*, 2005; Wu *et al*, 2009). PMCA4 has also been shown to interact with calcineurin in endothelial cells (Holton *et al*, 2010a) The aim of this project was to establish PMCA4s role in the regulation of the calcineurin/NFAT signalling pathway specifically in endothelial cells.

As the calcineurin/NFAT pathway has been implicated in the regulation of angiogenic processes (Hernández *et al*, 2001) the effect of PMCA4s regulation of the calcineurin/NFAT pathway will also be investigated in relation to angiogenesis, by studying the effect of ‘gain of function’ and ‘loss of function’ of PMCA4 on processes required for successful angiogenesis such as cell migration, tube formation and proliferation.

The finding that the interaction between PMCA and calcineurin is involved in the inhibitory function of PMCA on the calcineurin/NFAT signalling pathway (Buch *et al*, 2005; Baggott *et al*, 2012) which is hypothesised to maintain calcineurin in a low calcium microenvironment (Holton et al, 2010b) prompted us to establish the mechanism of PMCA4s regulation of the calcineurin/NFAT pathway and angiogenesis by investigating the role of the interaction between the two proteins on these processes.

We hypothesise PMCA4 is an inhibitor of the calcineurin/NFAT pathway in endothelial cells and consequently a negative-regulator of calcineurin-dependent processes involved in angiogenesis. We predict this novel role for PMCA4 will be the result of its interaction with calcineurin which is hypothesised to sequester calcineruin to a microdomain with a low calcium concentration generated by the calcium removal function of the pump. This study will investigate a novel role for PMCA4 in the regulation of angiogenesis and consider its potential therapeutically as a target in the treatment of diseases that are associated with pathological angiogenesis.

2. CHAPTER TWO

Materials and Methods

2.1 Tissue culture

All procedures and experiments requiring tissue culture were carried out in aseptic conditions using sterile equipment and techniques in a laminar tissue culture hood, to prevent the risk of contamination of cells.

2.1.1 Culturing Cells

All solutions required for tissue culture were pre-warmed to room temperature before use. Cells in culture were split to maintain and amplify the amount of cells for experiments. To do this, cells were washed with 1x phosphate buffered saline (PBS1x) and detached with 0.25% trypsin EDTA solution. When the cells had successfully detached, fresh complete medium was added to neutralise the trypsin and cells were centrifuged for 5 minutes at 1200 rpm. Pelleted cells were resuspended in fresh medium and transferred to tissue culture flasks. Cells were then incubated at 37ºC, 5% CO_2. The medium was changed every other day until cells reached confluency.

2.1.2 Isolation of Mouse Lung Endothelial Cells (MLEC)

Mouse lungs from 6-8 week old female mice were a generous gift from the Cardiovascular Research Group, University of Manchester, Manchester, United Kingdom. MLEC were isolated from both wild-type (*PMCA4*$^{+/+}$) and PMCA4 knockout mice (*PMCA4*$^{-/-}$). Lungs were washed in ethanol for 5 seconds and placed in MLEC medium (see section 2.1.3). Lungs were then minced into a

paste and digested in a 10 ml solution of Type I collagenase (Gibco, UK) (440 units/ml) in PBS1x pre-warmed at 37°C and then incubated for exactly one hour at 37°C, 5% CO_2. Fresh collagenase was prepared for each new round of endothelial cell isolation by incubating Type I collagenase in 25 ml PBS1x at 37ºC, 5% CO_2 for 1 hour. After this time an additional 25 ml PBS1x was added generating a final concentration of 440 units/ml. This solution was filter sterilised using a 0.2 µm filter and stored at 4ºC. After one hour incubation an equal volume of MLEC medium was added to the lung solution and the whole content was poured into a petri dish. Aggregates were disrupted by passing the solution through a 19 gauge needle four times and subsequent filtering through a 70 µm cell strainer into 20 ml of MLEC medium. The resulting cell solution was centrifuged for 5 minutes at 1200 rpm, the supernatant was removed and the pellet resuspended in 10 ml of MLEC medium and plated onto Primaria™ tissue culture dishes (BD biosciences, UK) that had been pre-coated for at least two hours with 0.1% gelatin, 0.1 mg/ml fibronectin from bovine plasma (SIGMA, UK) and 0.3 mg/ml collagen solution ultrapure bovine (SIGMA, UK) to aid attachment of the cells to the plate. The following day negative selection was carried out for the removal of macrophages and other immune cells that display the FcγIII/II receptor. The medium was removed and cells were washed with PBS1x. Fresh MLEC medium was added and incubated at 4°C for 20 minutes. The medium was aspirated off and replaced with 3 ml of a 0.0017 mg/ml solution of purified rat anti-mouse, CD16/CD32 (FcγIII/II receptor), antibody (BD Biosciences, UK) in PBS1x. Cells were incubated for 30 minutes at 4°C with regular rotation of the plate. This antibody solution was removed and 3 ml of a $6.67x10^6$ beads/ml solution of Dynabeads® sheep anti rat IgG (Invitrogen, UK)

in MLEC medium was added to the plate and incubated at 4°C for 30 minutes with regular turning of the plate. This solution was then removed and the cells were washed three times with PBS1x. Cells were trypsinised, and once detached from the plate, resuspended in MLEC medium and transferred in a centrifuge tube to a magnetic holder. After 5 minutes of allowing the beads to attach to the tube the cell solution was pipetted off and put into a pre-coated petri dish (see above) and incubated at 37°C, 5% CO_2. The beads were discarded. Medium was changed on the cells every day until colonies of approximately 20 cells were visible, at which point positive selection to isolate endothelial cells was carried out using an antibody directed against the endothelial cell marker ICAM-2. The same process as that used for negative selection was carried out with the following alterations; the antibody solution used in this case was 3 ml of a 0.0017 mg/ml CD102 purified rat anti mouse (BD Biosciecnes, UK) solution in PBS1x. Additionally, the beads containing attached endothelial cells were kept, resuspended in fresh MLEC medium and plated onto pre-coated petri dishes. Cells were incubated at 37°C, 5% CO_2 with the medium being changed every day until plates reached confluency. To assure a high percentage of endothelial cells (≥ 95%) in the culture a second round of positive selection with α-ICAM-2 was performed before using the cells for experiments. To establish the percentage of cells that were endothelial in the cultures, cells were viewed microscopically and counted. The number of endothelial cells, as identified by the attachment of beads, was calculated as a percentage of the total number of cells within the field. This was performed for several random microscopic fields to calculate an average percentage of endothelial cells in the population which we reported to be ≥ 95% in each case.

2.1.3 Mouse Lung Endothelial Cells (MLEC)

Isolated MLEC were plated on pre-coated plates (see section 2.1.2) and grown in MLEC medium complete consisting of 400 ml Dulbecco's Modified Eagle's medium (DMEM) F-12 medium (SIGMA, UK) with additional supplements, generating medium containing; 100 ml fetal bovine serum (FBS), 0.096 mg/ml heparin sodium salt from porcine intestinal mucosa (SIGMA, UK), 10 ml L-glutamine (200 mM) and 0.024 mg/ml endothelial cell growth supplement (AbD serotec, UK). An antibiotic/antimycotic solution (100x) was added to the medium resulting in a final concentration of 95.97 units/ml penicillin, 0.096 mg/ml streptomycin and 0.24 µg/ml amphotericin B. MLEC cells were used for experiments at very low passages (either when the cells reached confluency after positive selection (see section 2.1.2) or after one passage.

2.1.4 Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC supplied by TCS Cellworks, UK or PromoCell, UK were grown in tissue culture flasks pre-coated with 0.1% gelatin in endothelial cell growth medium (ECGM) complete consisting of 500 ml basal medium with an endothelial cell growth medium supplement mix (Promocell, UK) generating medium containing: 2% fetal calf serum, 0.4% endothelial cell growth supplement, 0.1 ng/ml epidermal growth factor (recombinant human), 1 ng/ml basic fibroblast growth factor (recombinant human), 90 µg/ml heparin and 1 µg/ml hydrocortisone. HUVEC cells between passages 7 and 10 were used for experiments.

2.1.5 Human Embryonic Kidney 293A Cells (HEK293A)

HEK293A cells were used to amplify the Ad-ID4 virus (see section 2.7.10). Cells were infected and grown in HEK medium consisting of 500 ml of DMEM (SIGMA life sciences, UK) supplemented with; 50 ml FBS, 1.73 mM L-glutamine, 17.39 mM HEPES buffer (SIGMA, UK), 5 ml non-essential amino acids (100x) and 5 ml of an antibiotic/antimycotic solution (100x) resulting in a final concentration of 86.96 units/ml penicillin, 0.087 mg/ml streptomycin and 0.22 µg/ml amphotericin B.

2.1.6 Serum Starvation

Where stated experiments required serum starvation. To do this cells were washed with PBS1x to completely remove any remaining medium containing serum and cells were then incubated in the corresponding medium with no growth factors, supplemented with 0.5% additional FBS. Cells were left overnight at 37ºC, 5% CO_2.

2.1.7 Counting Cells

After detachment of cells (see section 2.1.1) an aliquot of cell suspension was taken to count. This solution was pipetted onto a hemocytometer. The number of cells in a 16-square grid of the hemocytometer was then multiplied by 10 000 to get the number of cells in 1 ml of cell solution.

2.1.8 Freezing cells

Cells were detached and centrifuged to collect a pellet as stated in section 2.1.1. The supernatant was discarded and the pellet was re-suspended in 1 ml freezing solution (90% FBS and 10% dimethyl sulfoxide (DMSO)) and transferred to a freezing vial. Vials were then wrapped in tissue and stored at -

80ºC for a week to ensure slow freezing. After this time vials were transferred to liquid nitrogen (-180ºC) for long term storage.

2.1.9 Thawing cells

Vials were taken from liquid nitrogen and the cells were thawed quickly. Once thawed, the cell solution was added in stages to 9 ml of fresh medium complete to prevent damage to cells. The cells were centrifuged at 1200 rpm for 5 minutes to collect a pellet. The supernatant (containing DMSO) was removed and cells were re-suspended in fresh medium, transferred to an appropriate tissue culture flask and incubated at 37ºC, 5% CO_2.

2.2 Transfection and Infection of Cells

2.2.1 Electroporation Transfection

Electroporation for transfection of HUVEC with si-RNA non-target (si-NT) (Thermoscientific, Germany), that acts as a control as its sequence does not correspond to any known target, and si-RNA PMCA4 (Thermoscientific, Germany), that has the complementary sequence to PMCA4 (si-PMCA4) and knocksdown its expression, was carried out using an Amaxa™ HUVEC nucleofector™ kit (Lonza, UK) following the manufacturer's instructions. HUVEC (5×10^5 cells/cuvette) were transfected with the corresponding si-RNA (30 pmoles/cuvette) in HUVEC Nucleofector™ solution (Lonza, UK) using a Nucleofector® II (amaxa biosystems, UK) on programme A-034. Transfected cells were resuspended in fresh HUVEC medium complete and plated in 0.1%

gelatin pre-coated T-25 tissue culture flasks. The medium was changed after 24 hours incubation and cells used for experiments 48 hours after transfection.

2.2.2 Adenoviral Infection

All viruses were a generous gift from Dr Delvac Oceandy and Dr Tamer Mohamed from the Institute of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, UK with the exception of the Ad-ID4 virus which we generated as part of this project (see section 2.6 and 2.7 and results chapter 5 for Ad-ID4 virus production). HUVEC were plated in T-75 tissue culture flasks at a density of 2.5×10^6 cells/flask. For over-expression studies cells were infected with either Ad-LacZ (encoding the β-galactosidase protein) or Ad-PMCA4b (encoding human PMCA4b) at a MOI=50, for 72 hours at 37°C, 5% CO_2. To check the importance of the interaction between PMCA4b and calcineurin, HUVEC cells, seeded as above, were infected with Ad-LacZ or Ad-ID4 (encoding the region of human PMCA4 that interacts with calcineurin (428-651)) at a MOI=150 for 72 hours.

2.3 Protein Determination Assays

2.3.1 Immunoprecipitation (IP)

To determine infection with Ad-PMCA4 resulted in successful ectopic expression of PMCA4, HUVEC infected with either Ad-LacZ or Ad-PMCA4 in T-75 tissue culture flasks (see section 2.2.2) were washed with PBS1x. After the complete removal of PBS cells were lysed in 750 µl of radioimmunoprecipitation

assay (RIPA) buffer (1xPBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS 20 µM phenylmethylsulfanyl fluoride, 500 ng/ml leupeptin, 1.0 µg/ml aprotinin, 500 ng/ml pepstatin) and incubated on a shaker for 10 minutes at 4°C, after which time the lysate was collected using a cell scraper. Cell debris was removed by centrifugation at 13000 rpm for 3 minutes and to the supernatant 100 µl of washed protein A-agarose beads (Roche, UK) were added and incubated for 1 hour at 4°C on a rotator to pre-clear the lysates. Samples were then centrifuged for 2 minutes at 3000 rpm to remove the beads. Protein quantification was carried out using a BCA protein assay kit (Thermo Scientific Pierce, UK) following the manufacturer's instructions. Samples were adjusted to 2 mg/sample with RIPA buffer and 5 µl of 5F10 (an anti-pan PMCA mouse monoclonal antibody) (abcam, UK) and 40 µl of protein A-agarose beads were added and incubated overnight at 4°C with constant rotation. Beads were collected the following day by centrifugation at 3000 rpm for 2 minutes and washed in RIPA buffer 3 times. After complete removal of RIPA buffer, beads were resuspended in 2x NuPAGE® LDS sample buffer (Invitrogen, UK) including 5% of β-mercaptoethanol and stored at -20°C for western blot (see section 2.3.3).

2.3.2 Analysis of Protein Expression/Activation

Several experiments were carried out to analyse the expression/activation of various proteins. These are described as below and in addition summarised in Table 1.3 at the end of this section. Numbers in square brackets within the text correspond to the numbering of experiments within the table.

To determine the successful expression of 3xFlagPMCA4b(428-651) in HUVEC after infection with Ad-ID4 [1], HUVEC were seeded at a density of 3.5 x 10^5 cells/well and after overnight incubation at 37°C, 5% CO_2 infected with either Ad-LacZ or Ad-ID4 at a MOI=50 for 72 hours.

To analyse the expression levels of the proteins Cox-1, Cox-2 and RCAN1.4 [2], HUVEC were plated into 6-well tissue culture plates at a density of 3x10^5 cells/well and incubated at 37ºC, 5% CO_2, overnight.

The following day cells were infected with either Ad-LacZ or Ad-PMCA4b at a MOI=50 for 48 hours. Cells were then serum starved by incubation at 37ºC in ECGM (0.5% FBS) without additional growth factors for 16 hours. After serum starvation cells were stimulated with human VEGF-A_{165} (Peprotech, UK) (25 ng/ml) for 4 hours.

To study the effect of knockdown of PMCA4 on RCAN1.4 expression [3], HUVEC were transfected with the corresponding si-RNA (see section 2.2.1) and plated in a 6-well tissue culture plate. After 24 hours of incubation in medium complete, medium was changed for fresh medium and cells were incubated 24 hours more. The following day cells were serum starved overnight in 0.5% serum starvation medium after which time cells were stimulated for 4 hours with VEGF (25 ng/ml).

For the analysis of RCAN1.4 expression in MLEC [4], wild-type and knockout cells were plated at a density of 3.0 x 10^5 cells/well in a 6-well tissue culture plate. Cells were serum starved overnight by incubation in MLEC growth medium (0.5% FBS) without additional supplements. The following day cells were either left unstimulated or stimulated with VEGF (50 ng/ml) for 4 hours.

To analyse the effect of disrupting the interaction between PMCA4 and calcineurin on RCAN1.4 protein expression [5], HUVEC cells were seeded at a density of $3.5x10^5$ cells/well in a 6-well tissue culture plate. The following day cells were infected with Ad-LacZ or Ad-ID4 at a MOI=100 for 48 hours which was followed by overnight serum starvation in 0.5% medium. After this time cells were stimulated with VEGF (25 ng/ml) for 4 hours.

For Erk1/2 detection, HUVEC infected with the corresponding adenoviruses [6 and 7], or, transfected with si-NT or si-PMCA4 [8] were plated into tissue culture 6-well plates, starved in 0.5% serum starvation medium as described and left unstimulated or treated with VEGF (25 ng/ml) for 5 minutes.

In all cases cells were washed with PBS1x after stimulation to remove any remaining medium and stimulus, and lysed with 100 µl of 2x NuPAGE® LDS sample buffer (Invitrogen, UK) containing 5% of β-mercaptoethanol. Samples were heated at 100ºC in a heat block for 15 minutes and stored at -20ºC until analysed by western blot.

Experiment Number	Protein of Interest	Cells/well (6 well issue culture plate)	Transduced with Adenovirus or Transfected with si-RNA	Multiplicity of Infection (MOI)	Incubation with Virus (hours)	0.5 % Serum Starvation (16 hours)	VEGF (ng/ml)	Incubation with VEGF (time)
1	Successful expression of 3x FlagPMCA4b(428-651)(ID4) in HUVEC	$3.5x10^5$	Ad-LacZ or Ad-ID4	50	72	-	-	-
2	Expression levels of RCAN1.4, Cox-1 and Cox-2 in HUVEC with ectopic expression of PMCA4	$3x10^5$	Ad-LacZ or Ad-PMCA4b	50	48	Yes	25 ng/ml	4 hours
3	Expression levels of RCAN1.4 in HUVEC with knockdown of PMCA4	$3x10^5$	si-NT or si-PMCA4	-	-	Yes	25 ng/ml	4 hours
4	RCAN1.4 expression in $PMCA4^{+/+}$ and $PMCA4^{-/-}$ MLEC	$3x10^5$	-	-	-	Yes	50 ng/ml	4 hours
5	Expression levels of RCAN1.4 in HUVEC with overexpression of ID4	$3.5x10^5$	Ad-LacZ or Ad-ID4	100	48	Yes	25 ng/ml	4 hours
6	Activation of Erk1/2 in HUVEC with overexpression of PMCA4	$3x10^5$	Ad-LacZ or Ad-PMCA4b	50	72	Yes	25 ng/ml	5 minutes
7	Activation of Erk1/2 in HUVEC with ectopic expression of ID4	$3.5x10^5$	Ad-LacZ or Ad-ID4	100	72	Yes	25 ng/ml	5 minutes
8	Activation with kno		si-PMCA4					ninutes

Table 2.1: Summary of Experiments Performed to Analyse Protein Expression/Activation

2.3.3 Western Blot

To study protein expression sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out which separates proteins based on their molecular weight (MW). The gel consisted of two sections; the stacking gel was prepared by mixing:

- 2.50 ml of stacking gel buffer (0.5 M Tris-HCl, 0.4% SDS, pH6.6) (GENEFLOW, UK)
- 1.40 ml of Ultra Pure ProtoGel® 30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide (37.5:1) (acrylamide/bis-acrylamide) (GENEFLOW, UK)
- 6.10 ml of dH_2O
- 100 µl of 10% ammonium persulfate (APS)
- 60 µl of N,N,N',N'-Tetramethylethylenediamine (TEMED)

The resolving gel was prepared by adding:

- 3.75 ml of 10x resolving buffer (4x 1.5 M Tris-HCl, 0.4% SDS, pH8.8) (GENEFLOW, UK)
- X ml of Ultra Pure ProtoGel® 30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide (37.5:1) (acrylamide/bis-acrylamide) (GENEFLOW, UK)
- Y ml of dH_2O (up to a final volume of 14.06 ml)
- 100 µl of 10% ammonium persulfate (APS)
- 60 µl of N,N,N',N'-Tetramethylethylenediamine (TEMED)

In general proteins with a MW of less than 50 kDa were run in a 12% gel (5.60 ml acrylamide/bis-acrylamide and 4.55 ml dH_2O), those with a MW between 50 kDa and 100 kDa were run in an 8% gel (3.73 ml acrylamide/bis-acrylamide and 6.42 ml dH_2O) and proteins of 100 kDa or more were run in a 6% gel (2.80 ml acrylamide/bis-acrylamide and 7.35 ml dH_2O) (see appendix 1 for SDS-PAGE gel percentages for specific proteins). The resolving gel was added first into the PAGE equipment and butanol was pipetted on top to remove air bubbles and create an even surface to the gel. The gel was left to polymerise after which time butanol was removed by washing with dH_2O. The stacking gel was added on top of the resolving gel and left to polymerise. Protein samples were denatured by heating at 100ºC for two minutes, briefly incubated on ice to cool down and loaded into the gel. 12 µl of SeeBlue® markers (Invitrogen, UK) and 12 µl ProtoMarkers™ (National Diagnostics, UK) pre-stained proteins were included in the gel as markers of protein MW. Gels were run in running buffer (0.025 M Tris, 0.192 M Glycine and 0.1% SDS) (GENEFLOW, UK) at 150 volts for an hour and a half.

Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) Immobilon-P transfer membrane (Millipore, UK) for an hour and a half at 35 volts in transfer buffer (0.025 M Tris, 0.192 M Glycine, 20% methanol). After transferring, membranes were incubated for 1 hour, or overnight in the case of membranes used to detect 3xFlag, at 4ºC in a 5% w/v solution of skimmed milk in tris buffered saline (TBS) 1x to block unspecific binding of antibodies to the membrane. Membranes incubated for 1 hour were briefly washed in TBS with 0.05% Tween 20 (TBS-T) to remove the milk solution and incubated overnight at 4ºC with shaking in the appropriate primary antibody solution (see appendix 1). The following day membranes were washed for 4 minutes, 5 times with TBS-T to remove any unbound antibody and then incubated at room temperature for an hour and a half in the appropriate secondary antibody (see appendix 1) or in the case of membranes for the detection of 3xFlag briefly washed with TBS-T and incubated in the primary antibody, anti-Flag M2 peroxidase (HRP) conjugate antibody (SIGMA, UK), for three hours. In all cases both primary and secondary antibodies were diluted in TBS-T. All secondary antibodies and the primary anti-Flag antibody were conjugated to the enzyme horseradish peroxidase (HRP), for detection using chemiluminescence. After incubation in the secondary antibody (or primary antibody in the case of detection of 3xFlag) membranes were washed as above and incubated for 1 minute in chemiluminescence detection solution prepared by mixing the components of an EZ-ECL chemiluminescence detection kit (GENEFLOW, UK) in a 1:1 ratio (1 ml solution A: 1 ml solution B). Chemiluminescence signal was recorded by exposure to Kodak BioMax MS auto-radiographic film and subsequent development.

2.4 RNA Determination

2.4.1 RNA Extraction

To determine the effect of over-expression of PMCA4 on the RNA levels of RCAN1.4 and Cox-2, HUVEC cells were seeded at a density of $3x10^5$ cells/well in a 6-well tissue culture plate and incubated at 37°C, 5% CO_2. The following day cells were infected with Ad-LacZ or Ad-PMCA4b at a MOI=50 for 48 hours, after which time cells were serum starved overnight in 0.5% serum. Cells were then stimulated with VEGF (25 ng/ml) for 2 hours and RNA was extracted. To determine if knockdown of PMCA4 by si-RNA transfection at the RNA level was successful transfected cells (see section 2.2.1) were plated in 6-well tissue culture plates at a density of $3.5x10^5$ cells/well and the following day RNA was extracted. In both cases RNA was isolated using a total RNA purification kit (Norgen, UK). All centrifugation steps were carried out for 1 minute at 13000 rpm unless otherwise stated. The medium was removed and cells were lysed in 300 µl of lysis buffer after which time 200 µl of ethanol (100%) was added, samples were vortexed for 10 seconds, transferred to columns and centrifuged. A DNase I solution was prepared by mixing DNase I (Norgen, UK) and enzyme incubation buffer in a 1:6.67 ratio (15 µl:100 µl). 100 µl of this solution was added per column and centrifuged. The DNase I solution was then pipetted back onto the column and left for 15 minutes at room temperature. After this time, 400 µl of wash solution was added to the column and centrifuged and this step was repeated. Samples were then centrifuged once more to completely eliminate the wash buffer. RNA was eluted by addition of 50 µl of RNA elution buffer and incubation for 1 minute at room temperature. Samples were then

collected by centrifugation for 2 minutes at 2000 rpm followed by centrifugation for 1 minute at 13000 rpm after which samples were stored at -80°C.

2.4.2 RNA Quantification

The RNA concentration (ng/µl) of each sample was calculated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK) by loading 1.5 µl of the sample onto the disc. The purity of the sample was also checked by looking at the $A_{260/280}$ and $A_{260/230}$ ratios.

2.4.3 Reverse Transcription

Reverse transcription to convert RNA into cDNA was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK). After quantification, samples were prepared by adjusting all to 500 ng in a final volume of 9 µl with nuclease-free water (Promega, UK). Adjusted samples were heated for 10 minutes at 65°C after which time they were put directly to ice for at least 5 minutes. A master mix was prepared containing a final concentration per sample of; 1x RT buffer, 1x dNTP mix (4 mM), 1x RT random primers, RNase inhibitor (1 unit/µl), MultiScribe reverse transcriptase (5 units/µl) and nuclease free water up to a volume of 11 µl. This master mix was added to each sample generating a final volume of 20 µl. Samples were then put in a thermal cycler (Thermo Hybaid, UK) and reverse transcription carried out under the following conditions; 42°C for 60 mins, 52°C for 30 mins, 94°C for 3 mins and 4°C until ready to collect. These samples were then used for real-time polymerase chain reaction.

2.4.4 Resuspension of Oligonucleotides

All oligonucleotides (SIGMA, UK) were resuspended in autoclaved dH_2O and the concentration (µg/µl) measured in a Jenway Genova machine using the photometrics setting at a wavelength of 260 nm. $1OD_{260}$ in this case correlates to 20 µg as the oligonucleotides were single stranded. Resuspended oligonucleotides were then adjusted to a final concentration of 100 ng/µl.

2.4.5 Semi-Quantitative Polymerase Chain Reaction (PCR)

PCR amplification was used to determine the successful knockout of PMCA4 in MLEC. MLEC PMCA4$^{+/+}$ and PMCA4$^{-/-}$ cells were plated at a density of $3x10^5$ cells/well in pre-coated 6-well tissue culture plates. The following day cells were washed in PBS1x, lysed in 300 µl of RNA lysis buffer (Norgen, UK) and RNA extraction and reverse transcription carried out as previously described (see section 2.4.2 and 2.4.3 respectively). Reverse transcription was performed after adjustment of the samples to 1 µg of RNA. PCR was then carried out on the reverse transcribed samples using oligonucleotides for mouse PMCA4 (mRNA). PMCA1 mRNA was also studied as a control using oligonucleotides for mouse PMCA1 (mRNA) and the housekeeping gene, *Hprt1* detected as a control using the mouse Hprt1 (mRNA) oligonucleotides (see appendix 2 for all oligonucleotides). All PCR reactions contained the following; PCR template, forward oligonucleotide (2 ng/µl), reverse oligonucleotide (2 ng/µl), 1x Roche PCR master mix (high fidelity PCR master mix (Roche, UK) and PCR water (H_2O PCR grade) (Roche, UK) up to a final volume of 50 µl. Samples were then amplified using the following cycles: 94ºC for 2 minutes (initial denaturation), 35 cycles of 94ºC for 1 minute (denaturation), the required annealing temperature for 1 minute (see appendix 2) and 72ºC for 1 minute 30 seconds. Samples were finally incubated at 72ºC for 10 minutes (final elongation). The melting

temperatures (Tm) were calculated using the formula Tm= 2(A+T) + 4(G+C). Annealing temperatures used were Tm minus 4°C. Bands were then viewed by running amplified samples in a DNA agarose gel (see section 2.7.4) (see appendix 2 for size of bands and gel percentages).

2.4.6 Real-Time Polymerase Chain Reaction (Real-Time PCR)

To begin cDNA samples from reverse transcription were diluted 1/10 with nuclease-free water (Promega, UK). To a MicroAmp® fast optical 96-well reaction plate (Applied Biosystems, UK) 5 µl of cDNA sample/well (1.25 ng/µl) was added. A master mix was prepared containing a final concentration/well of; 1x syber green (Primerdesign, UK), 0.25 µM forward primer, 0.25 µM reverse primer (see appendix 3 for specific primer sequences) and nuclease free water up to a volume of 15 µl, and was added to the samples generating a final volume of 20 µl/well. Samples were then run in a Stratagene MX3000 system at FAM490 wavelength using the following conditions; 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The (cycle threshold) C_t value was recorded and normalised using the C_t values for two housekeeping genes; Ubiquitin C (UBC) and YWHAZ. These values were then used to calculate the fold change of RNA levels using the $2^{-\Delta\Delta Ct}$ method of analysis (see section 2.8.2). Melting curves were also carried out to ensure no primer dimers had been formed and that specific amplification had occurred.

2.5 Functional Assays

2.5.1 Calcineurin/NFAT Pathway Activity

To study the effect of over-expression of PMCA4 on the activity of the calcineurin/NFAT signalling pathway HUVEC were seeded at a density of $3.5x10^5$ cells/well in a 6-well tissue culture plate. Cells were then infected with Ad-LacZ or Ad-PMCA4 at a MOI=50 for 24 hours. After this time Ad-NFAT-Luc (a NFAT-dependent luciferase-based reporter vector) was added to the cells at a MOI=50 and cells were incubated for 24 additional hours. Cells were serum starved in 0.5% serum starvation conditions overnight. The following day cells were stimulated with VEGF (25 ng/ml) for 6 hours.

MLEC cells were plated into a 24-well tissue culture plate at a density of $6.5x10^4$ cells/well or in a 6-well plate at a density of $3x10^5$ cells/well. The following day cells were infected with Ad-NFAT-Luc at a MOI=50 for 48 hours after which time cells were serum starved in 0.5% serum overnight. Cells were then stimulated with phorbol-12 myristate 13-acetate (PMA) (20 ng/ml) and Ionophore A23487 (1 μM) for 6 hours.

To study the effect of disruption of the PMCA/calcineurin interaction on NFAT activity, HUVEC were plated in 6-well tissue culture plates at a density of $3.5x10^5$ cells/well. The following day cells were infected with Ad-LacZ or Ad-ID4 at an MOI=150. After overnight incubation, Ad- NFAT-Luc was added at a MOI=25 and cells were incubated at 37°C, 5% CO_2 for an additional 24 hours. The next day cells were serum starved using 0.5% serum starvation conditions. After this time cells were stimulated with VEGF (25 ng/ml) for 6 hours.

In all cases after stimulation, cells were washed with PBS1x and lysed in 60 μl cell culture lysis reagent 1x (Promega, UK) for 10 minutes at room temperature. Luciferase activity in the samples was determined by measuring 20 μl of the cell

lysate in a SIRIUS Luminometer V3.1 (Berthold detection systems, UK) that injected 100 µl of luciferase assay substrate from a luciferase assay system kit (Promega, UK) into the samples and recorded the amount of relative light units (RLU) produced after 30 seconds.

2.5.2 Cell Migration

Cell migration assays were carried out using 24-well cell culture cluster plates (COSTAR®, UK). Infected cells for over-expression studies (see section 2.2.2) were plated at a density of 5 x 10^4 cells/well and a 0.9 mm gap was created using inserts from a Cytoselect™ 24-well wound healing assay kit (Cell Biolabs Inc, UK). The following day cells corresponding to time zero (t=0) were stained with a 0.6% crystal violet/0.025% ammonium oxalate/5% ethyl alcohol solution (SIGMA-ALDRICH, UK) for 15 minutes at 37°C. Excess stain was removed by washing with PBS1x three times and cells were fixed at room temperature for 10 minutes using formalin solution, 10% neutral buffered containing formaldehyde 4% w/v (SIGMA-ALDRICH, UK). Fixed cells were further washed twice with PBS1x and visualised using a microscope Nikon ECLIPSE TS100 at 4x magnification. Images were recorded using a digital camera (Nikon DSFi1, UK). The remaining cells were incubated in ECGM complete supplemented with VEGF (25 ng/ml) for 24 hours (t=24). After incubation, cells were stained, fixed and viewed as above.

MLEC migration was studied using the same protocol as previously mentioned apart from the following alterations; MLEC were directly plated (no virus infection) at a density of 1 x 10^5 cells/well into pre-coated (see section 2.1.2) 24-

well plates. Cells were also incubated in MLEC medium complete supplemented with VEGF (50 ng/ml) for 24 hours.

To calculate the percentage of migration, the unmigrated area was determined using the Image J programme and compared to the unmigrated area of time zero (taken as 100%). The difference between the total area at time zero and the unmigrated area after stimulation with VEGF represents the percentage of migration.

2.5.3 Tube Formation

In all cases the appropriate tissue culture plates were pre-coated with Geltrex™ Matrix (low growth factors) (GIBCO®, UK) and incubated at 37ºC for 30 minutes to allow the gel to set up.

For over-expression studies, infected cells (see section 2.2.2) were detached, centrifuged for 5 minutes at 1200 rpm to pellet cells and washed in Medium 200 (Cascade Biologics™, UK). Cells were pelleted again by centrifugation, re-suspended in Medium 200 and plated onto geltrex at a density of $1x10^5$ cells/well in a 24-well tissue culture plate in either Medium 200 with 2% FBS (control) or Medium 200 with 2% FBS supplemented with VEGF (50 ng/ml) and incubated for 24 hours at 37°C.

For PMCA4 knockdown studies HUVEC si-RNA transfected cells (see section 2.2.1) were plated in a pre-coated 96-well tissue culture plate at a density of $3x10^4$ cells/well in Medium 200 with 2% FBS or 2% FBS plus additional VEGF (50 ng/ml) and incubated for 24 hours.

HUVEC cells infected with Ad-LacZ or Ad-ID-4 (see section 2.2.2) to determine the effect of disruption of the interaction between PMCA4 and calcineurin on tube formation, were plated in geltrex pre-coated 96-well tissue culture plates at a density of $3x10^4$ cells in Medium 200 (Cascade Biologics™, UK) with additional 2% FBS. Cells were then incubated for 24 hours at 37°C, 5% CO_2.

In all cases cells were fixed after the appropriate incubation time with formalin solution, 10% neutral buffered containing formaldehyde 4% w/v (SIGMA-ALDRICH, UK) at 37ºC, 5% CO_2 for 15 minutes. The fixative was then removed and replaced with PBS1x and cells were viewed using a Nikon ECLIPSE TS100 microscope and Nikon DSFi1digital camera at 4x magnification. The percentage of tube formation was determined by counting branching points from the images taken. The value for cells infected with Ad-LacZ and incubated with 2% serum was set as 100% and used as the control. Values for all the other conditions were calculated as a percentage in relation to the control.

2.5.4 MTT Assay-Proliferation

Infected cells for over-expression studies (see section 2.2.2) were plated at a density of $5x10^3$ cells/well in three tissue culture 96-well plates and incubated overnight at 37ºC. The following morning cell number at time zero (t=0) was determined in one of the plates by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) analysis by adding 50 µl/well of 5 mg/ml MTT directly to the culture medium and subsequent incubation for 4 hours in the dark at 37ºC. After incubation, medium containing MTT was removed and 175 µl of DMSO and 25 µl of Sorensen's glycine buffer (0.1 M glycine and 0.1 M NaCl

equilibrated to pH10.5) was added to each well. The absorbance of the resulting solution was measured at 540 nm in a Multiskan Ascent (ThermoLabsystems, UK). In the other two plates, the medium was replaced with medium ECGM complete containing VEGF (25 ng/ml) and cells were further incubated at 37ºC, 5% CO_2 and analysed after three days (t=3) by standard MTT assay. The medium was changed on the other three plates and analysed after 6 days (t=6) as above.

2.6 Microbiology

2.6.1 Preparation of Luria Broth and Antibiotic Plates

Luria broth (LB) was prepared by dissolving 25 g of luria broth (SIGMA life sciences, UK) in a final volume of 1000 ml. These were aliquoted into 400 ml and autoclaved. LB agar for the production of plates was made by addition of 6 g of agar (Fluka BioChemika, UK) to freshly prepared (before autoclaving) 400 ml aliquot of LB generating a final concentration of 0.015 g/ml and then autoclaved. Autoclaved agar was melted using a microwave and poured into petri dishes 25 ml/plate containing 100 µg/µl of the appropriate antibiotic for selection.

2.6.2 Mini-Preparations

Colonies formed after bacterial transformation (see section 2.7.2), were picked up and added to 10 ml of LB containing 100 µg/ml of the appropriate antibiotic using aseptic conditions and incubated at 37°C with shaking. The following day mini-preparations were carried out using a QIAprep® Miniprep kit (QIAGEN, UK). All centrifugation steps were carried out at 13000 rpm unless otherwise

stated. Cultures were centrifuged for 10 minutes at 4000 rpm and the supernatant was discarded. Pellets were resuspended in 250 µl of buffer P1 (resuspension buffer) and then an equal volume of buffer P2 (lysis buffer) was added and mixed by inversion. Additionally, 350 µl of buffer N3 (neutralisation buffer) was added and mixed by inversion. Samples were centrifuged for 10 minutes then the supernatant was transferred to a QIAprep® spin column. Centrifugation for 1 minute was followed by addition of 750 µl of buffer PE (wash buffer) to the column. Samples were centrifuged for 1 minute then centrifuged in the same conditions again to completely eliminate the wash buffer. Samples were eluted in 30 µl of buffer EB (elution buffer) by incubation for 1 minute at room temperature followed by centrifugation for 2 minutes.

2.6.3 Maxi-Preparations

Before maxi-preparations were preformed starter cultures of plasmid were produced containing 10 ml of LB, the required plasmid and 100 µg/ml of the appropriate antibiotic and incubated at 37°C with shaking overnight. The following morning 1 ml of the starter culture was added to 9 ml of LB with 100 µg/ml of antibiotic and incubated at 37°C until the evening, to ensure that bacteria were in the log phase of growth, and were then inoculated into 400 ml bottles of LB plus antibiotic. Inoculated plasmids were incubated overnight with shaking at 37°C. The following day maxi-preparations of plasmids were carried out using a plasmid maxi kit (Qiagen, UK). Cultures were centrifuged at 4500 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml buffer P1 (resuspension buffer) containing RNase A. An equal volume of buffer P2 (lysis buffer) was added and mixed by inversion. The same was done

for buffer P3 (neutralisation buffer). Solutions were centrifuged for 15 minutes at 4500 rpm and columns were prepared by adding 10 ml of buffer QBT (equilibration buffer). After centrifugation the supernatant was pipetted into the columns and once it had run through, 30 ml of buffer QC (wash buffer) was added. An additional 30 ml of wash buffer was added and once this had passed through the column the column was transferred to a centrifuge tube and the plasmid was eluted by addition of 15 ml of buffer QF (elution buffer). After this step, 10.6 ml of 2-propanol (Isopropanol) was added and mixed by inversion. The plasmid was then centrifuged at 9600 rpm for 1 hour at 4°C. After this time the supernatant was discarded and the pellet resuspended in 300 µl of Tris-EDTA buffer 1x (TE1x). Samples were centrifuged at 13000 rpm for 1 minute to remove any debris. The concentration (µg/µl) was calculated by measuring the absorbance of the sample at 260 nm using the photometrics setting that correlates $1OD_{260}$ to 50 µg in a Jenway Genova machine.

2.7 Cloning

2.7.1 PCR Amplification

PCR amplification of the human PMCA4b interaction region (428-651) including a Methionine start codon and 3 copies of the Flag-epitope was carried out using the pF-PMCA4b-(428-651) vector previously generated by Buch et al, 2005 as a template (Buch *et al*, 2005) and the oligonucleotides BamHIPMCA4b Sense and XhoIPMCA4b Anti-sense (see appendix 2 for oligonucleotides). The sense

oligonucleotide sequence is complementary to the sequence just upstream of the start codon ensuring that the 3xFlag is incorporated in the amplified product and also contains a BamHI restriction site. The anti-sense oligonucleotide sequence is complementary to the end of the interaction region (428-651), includes a stop codon and also a XhoI restriction site. These restriction sites were selected as they are not found within the fragment that is being amplified and could therefore be used later on in the cloning process. PCR amplification reactions were carried out as stated in section 2.4.5 (see appendix 2 for annealing temperatures) and run in a DNA agarose gel (see section 2.7.4)

2.7.2 Transformation

pGEM®-T- 3xFlagPMCA4b(428-651) and pENTR™ 11- 3xFlagPMCA4b(428-651) were transformed in 200 µl of JM109 competent cells (Promega, UK). After addition to bacteria, samples were incubated on ice for 30 minutes after which they were heat shocked at 42°C for 2 minutes. LB was then added up to a final volume of 500 µl and incubated at 37°C with shaking for 1 hour. The pGEM®-T-3xFlagPMCA4b(428-651) and pENTR™ 11- 3xFlagPMCA4b(428-651) samples were then plated onto ampicillin and kanamycin LB agar (100 µg/ml) plates respectively and incubated overnight at 37°C.

2.7.3 Restriction Digests

All restriction digest enzymes and their corresponding buffer were bought from Promega, UK apart from PacI and its relating buffer which was from New England BioLabs, UK (see appendix 4 for a table of enzymes and the appropriate buffer). Reactions were set up as follows, plasmid (sample), restriction enzyme, buffer, 1x bovine serum albumin (BSA) (New England

BioLabs, UK) and dH_2O up to a final volume of 20 µl. Initially only half of the required amount of restriction enzyme was added to the sample and incubated at 37°C for 2 hours after which time the remaining amount of enzyme was added and incubated overnight at 37°C. In the case of mini-preparation digests, samples were incubated with the full amount of enzyme for 2.5 hours.

2.7.4 Agarose Gel Electrophoresis

DNA agarose gels were made to the appropriate percentage dependent on the size of the product that was being observed (see appendix 4 for table of percentages of gel and size of fragments). In general DNA with a size below 500 bp was run in a 2% gel, between 500 bp-1000 bp in a 1% gel and those above 1000 bp in a 0.7% gel. Gels were made by dissolving the required amount of agarose, electrophoresis grade (Invitrogen, UK) in a total volume of 60 ml Tris-Acetate-EDTA (TAE) 1x buffer (Invitrogen, UK) in a microwave. After allowing the gel solution to cool slightly, ethidium bromide (Invitrogen, UK), final concentration $5x10^{-4}$ mg/ml, was added and poured into the casting gel device. 15 µl of a 1kb ladder solution (0.17 µg/µl) (Invitrogen, UK) was loaded as a marker of DNA size. Gel loading solution 6x was added to the samples at a final concentration of 1x allowing samples to be loaded and retained in the gel. Samples were run at 80 volts for 30 minutes and then viewed using UV light in a SYNGENE Bio imaging machine (Geneflow, UK).

2.7.5 Purification of DNA From Agarose Gels

To purify DNA from agaose gels, samples were run in the appropriate percentage gel (see appendix 2 and 4) and the required band was viewed and selected using UV light on a UV Transilluminator 2000 (BIORAD, UK) and cut

from the gel. DNA was extracted and purified from the gel using a QIAquick® gel extraction kit (QIAGEN, UK). All centrifugation steps were carried out for 1 minute at 13000 rpm. 3 volumes of buffer QG (solubilisation buffer) was added to the samples and they were incubated at 50°C for 10 minutes, with vortexing every 2 minutes until the gel had completely dissolved. 1 volume of isopropanol was added, inverted to mix and the sample was transferred to a QIAprep® spin column and centrifuged. 500 µl of buffer QG was added to the column and centrifuged. 750 µl of buffer PE (wash buffer) was added to the column and left for 5 minutes followed by centrifugation. Centrifugation was carried out again to ensure complete removal of the wash buffer. The DNA was then collected in 30 µl of buffer EB (elution buffer) by addition of it to the column, 4 minute incubation at room temperature and centrifugation. Samples were then stored at -20°C.

2.7.6 DNA Precipitation

For DNA precipitation reactions, 10% of sodium acetate (NaAc) 3 M solution and 2.5 volumes of ice cold ethanol (100%) were added to the sample and stored at -20°C overnight. After this time, precipitated DNA was collected by centrifugation at 13000 rpm for 15 minutes, the supernatant was removed and DNA resuspended in an appropriate buffer.

2.7.7 Adenylation

Adenylation was used to add the nucleoside, deoxyadenosine, to the 3’ end of DNA, in this case the 3xFlagPMCA4b(428-651) fragment to allow cloning into the pGEM®-T Easy Vector (Promega, UK) that has 3’ thymidine to ensure successful ligation. Precipitated DNA that had been collected and resuspended

in 20 µl of PCR water (Roche, UK) was used to set up adenylation reactions using the following components; template (precipitated DNA) (0.651 µg), 1x Taq buffer (Mg free) (Promega, UK), $MgCl_2$ (2.5 nM), dNTPs (0.8 mM each), aTaq DNA polymerase (0.1 units/µl) (Promega, UK) and PCR water up to a final volume of 50 µl. Reactions were run for 30 minutes at 72°C in a thermal cycler.

2.7.8 Ligation

Ligation procedures were carried out to join the insert, adenylated 3xFlagPMCA4b(428-651), into the pGEM®-T Easy Vector (Promega, UK) and to insert the purified released fragment from the pGEM®-T- 3xFlagPMCA4b(428-651) into the pENTR™ 11 dual selection vector (Invitrogen, UK) after both had been digested with XhoI and BamHI. Ligation reactions were set up as follows; vector, insert, T4DNA ligase (400 u/µl) (New England biolabs, UK), 1x ligase buffer (New England biolabs, UK) and dH_2O up to a final volume of 10 µl. Samples were incubated overnight at 15°C in a thermal cycler.

2.7.9 Recombination

The pENTR™ 11-3xFlagPMCA4b(428-651) was recombined into the pAd/CMV/V5-DEST™ vector (Invitrogen, UK). Reactions were set up as follows; 300 ng of each plasmid, TE buffer and 2 µl of Gateway® LR Clonase™ II enzyme mix (Invitrogen, UK) up to a final volume of 10 µl. Samples were incubated at 25°C for 5 hours after which time the sample was transformed (see section 2.7.2) in DH5α competent bacteria. The bacteria were then plated onto ampicillin (100 µg/ml) LB agar plates and incubated overnight at 37°C.

2.7.10 Virus Amplification and Collection

Dr Mohamed and Dr Delvac from the Institute of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, UK, kindly generated and amplified the adenoviruses used in this study. The Ad-ID4 virus was generated from adenoviral plasmids digested with PacI as stated in Mohamed *et al*, 2010 with the following exceptions: Tertiary stock of the virus was generated by infection of HEK293A cells with the secondary stock of virus (30 µl/flask) for 3 days until all cells had become rounded and detached. Cells were collected and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was washed in 5 ml PBS1x with great care. Cells were re-centrifuged at 1500 rpm for 5 minutes, the supernatant was removed, and the pellet resuspended in 0.5 ml/flask of PBS1x. Purification of the virus was performed by adding an equivalent volume of chloroform and mixing cells continuously by inversion for 5 minutes after which time the solution was centrifuged for 15 minutes at 3000 rpm. The top phase (containing virus) was collected and stored at -80°C.

2.7.11 Sequencing

The pAd/CMV/V5-DEST™- pENTR™ 11-3xFlagPMCA4b(428-651) (pAd-ID4) plasmid was sequenced by the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares CNIC, Madrid, Spain. Sequencing of pAD-ID4 confirmed the fidelity of the PCR amplification and the successful recombination and generation of the plasmid for Ad-ID4 adenovirus production (see appendix 5).

2.8 Data Analysis

2.8.1 Protein Quantification

For analysis of protein expression, the density of bands from western blot images was calculated using Image J software. The value obtained for Ad-PMCA4 tubulin was set as 1 and the density for Ad-LacZ tubulin normalised relative to this. Normalisation of the density of the bands for the identified proteins was carried out by dividing the density values of the specific protein by the corresponding adjusted tubulin values. Ad-LacZ normalised results were then set as 100% and normalised data for Ad-PMCA4 calculated as a percentage relative to this.

2.8.2 RNA Quantification

To analyse the RNA levels of the gene of interest (GOI), samples were firstly normalised to obtain the ΔC_t value by subtracting the C_t values for the housekeeping genes (HKG) from the C_t values for the equivalent sample assayed for the gene of interest ($\Delta C_t = C_t$ GOI - C_t HKG). The alterations in RNA levels between samples were then calculated in respect to a reference which was si-NT in Fig. 3.3.1 and Ad-LacZ unstimulated (-) in Fig. 4.2.2. To do this the ΔC_t of the reference was subtracted from itself and from the ΔC_t of all samples giving the $\Delta\Delta C_t$ value ($\Delta\Delta C_t = \Delta C_t$ Sample - ΔC_t Reference). $2^{\wedge -\Delta\Delta Ct}$ expresses the data as the fold change of the samples, as a result the reference gene becomes 1 and all other samples calculated as a fold change relative to the reference (Fold change = $2^{\wedge - \Delta\Delta Ct}$).

3. CHAPTER THREE

Results

Role of PMCA4b in the Regulation of the Calcineurin/NFAT Pathway in Endothelial Cells

3.1 Introduction

It has previously been reported by our group that PMCA4 interacts with calcineurin, via PMCAs large intracellular loop between transmembrane domains TM4 and TM5, in mammalian HEK293 cells (Buch *et al*, 2005), breast cancer MCF-7 cells (Holton *et al*, 2007) and endothelial cells (Holton *et al*, 2010a). Additionally, ectopic expression of human PMCA4b has been shown to down-regulate NFAT activity and therefore act as a negative regulator of the calcineurin pathway in HEK293 cells (Buch *et al*, 2005). Thus our first aim was to determine if this effect also occurred in endothelial cells. To do this we carried out 'gain of function experiments' by over-expressing PMCA4b and

determining its effect on the activity of the calcineurin/NFAT pathway. We hypothesised that over-expression of PMCA4b would result in inhibition of endogenous calcineurin and consequently the activity of the calcineurin/NFAT pathway (Fig. 3.1.1).

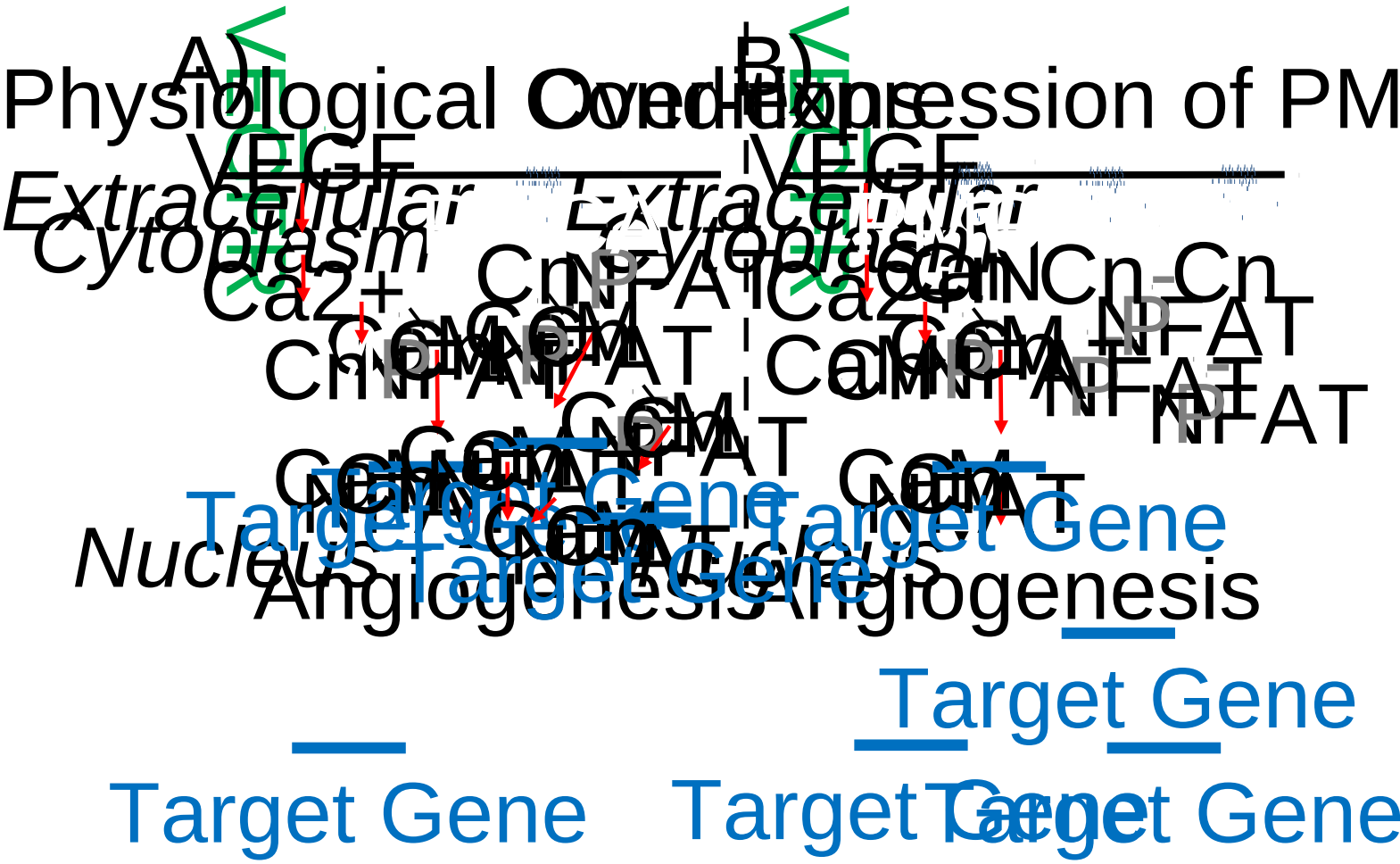


Fig. 3.1.1 Hypothesis of PMCAs down-regulation of the calcineurin/NFAT pathway and consequently angiogenesis. A) Under physiological conditions calcineurin (Cn) is activated by calmodulin (CaM) in response to VEGF binding to its receptor (VEGFR) resulting in an increase in the concentration of cytosolic calcium. Activated calcineurin then interacts with the nuclear factor of activated T-cells (NFAT) resulting in its dephosphorylation and translocation to the nucleus. Here it binds to promoters on target genes and initiates their transcription, in this case, those involved in angiogenesis resulting in an increase in angiogenic processes. B) With over-expression of PMCA4 we hypothesise more calcineurin will interact with PMCA4 resulting in its sequestration in a low calcium microenvironment (represented by the grey area at the plasma membrane) and will inhibit its activity. As a consequence NFAT will not be dephosphorylated, will not translocate to the nucleus and will not activate gene transcription, therefore resulting in inhibition of angiogenesis.

3.2 Over-Expression of PMCA4b Using Adenovirus and its Effect on the Calcineurin/NFAT Pathway

To ectopically over-express PMCA4b in endothelial cells, HUVEC were infected with an adenovirus encoding PMCA4b (Ad-PMCA4) or the control LacZ (Ad-LacZ). PMCA proteins from infected cells were immunoprecipitated using the anti-pan PMCA 5F10 monoclonal antibody. Western blot of the immunoprecipitated proteins using an antibody recognising specifically PMCA4 (Swant, Switzerland) demonstrated PMCA4 over-expression had been successful in cells infected with Ad-PMCA4 compared to the control (Fig. 3.2.1,

A, upper panel). In addition to this a western blot detecting an anti-PMCA1 antibody (Swant, Switzerland) was also completed to show that ectopic expression of PMCA4 was specific and had no effect on the expression of other PMCA isoforms (Fig. 3.2.1, A, lower panel). As the ectopic expression of PMCA4 in HUVEC had been successful we next went on to study the effect of PMCA4 on the regulation of the calcineurin/NFAT pathway. HUVEC cells were infected as above in conjunction with an adenovirus encoding NFAT luciferase (Ad-NFAT-Luc). Luciferase activity is used as a surrogate measurement of the activity of the calcineurin/NFAT pathway. Infected HUVEC were stimulated with VEGF (25 ng/ml) for 6 hours to activate the pathway. The presence of ectopic PMCA4 resulted in a reduction in the percentage of NFAT activation by 31.92% compared to the NFAT activity in control infected cells (Fig. 3.2.1, B).

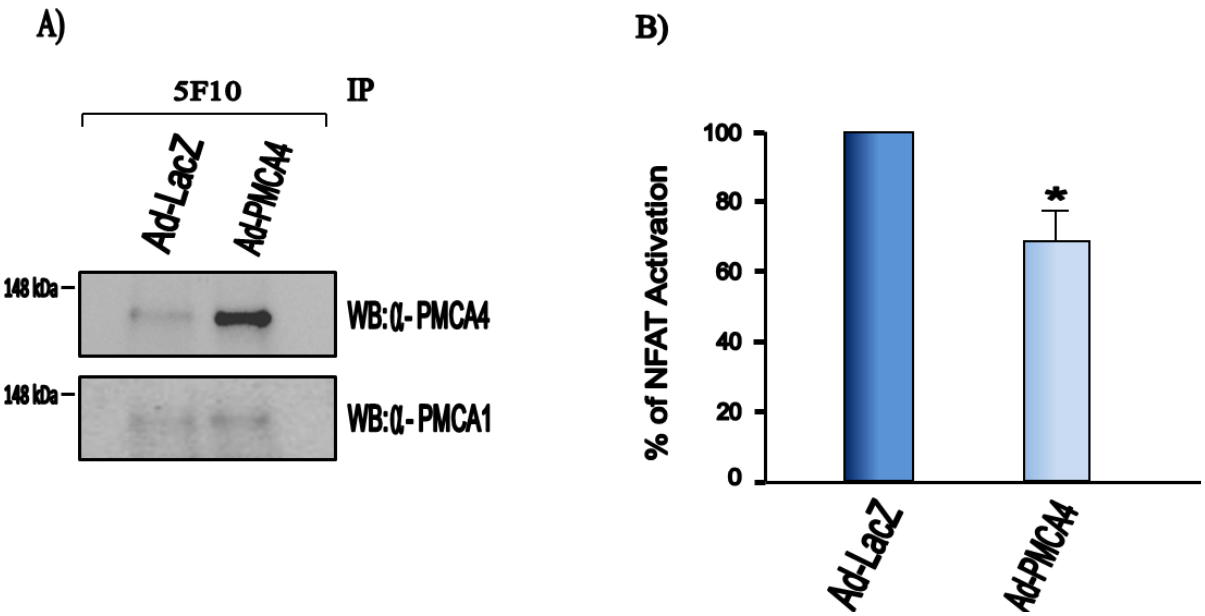


Fig. 3.2.1 VEGF-stimulation of the calcineurin/NFAT pathway is inhibited by adenoviral expression of PMCA4 in HUVEC. A) Ectopic expression of PMCA4 by adenoviral-mediated infection of HUVEC. HUVEC were infected with either Ad-LacZ or Ad-PMCA4b and protein lysates immunoprecipitated with the anti-PMCA 5F10 antibody analysed by western blot to detect PMCA4 (WB: α-PMCA4) and PMCA1 (WB: α-PMCA1). B) Ectopic expression of PMCA4 significantly decreased the activity of NFAT. Cells were infected as above, in conjunction with Ad-NFAT-Luciferase and stimulated with VEGF (25 ng/ml) for 6 hours, after which, luciferase activity was measured. Histogram represents mean ± S.E. of 2 independent experiments, n=5 (* $p \leq 0.05$).

3.3 Knockdown and Knockout of PMCA4 and its Effect on the Calcineurin/NFAT Pathway

3.3.1 Si-RNA Mediated Knockdown of PMCA4 in HUVEC

In the case of the knockdown, HUVEC were transfected with si-PMCA4 or the control si-NT. Successful knockdown of PMCA4 was confirmed by western blot with protein lysates obtained from si-PMCA4 transfected cells using the JA3 anti-PMCA4 monoclonal antibody (Santa Cruz, UK). Protein lysates of HUVEC cells transfected with si-NT were used as a control (Fig. 3.3.1, A, upper panel). Additionally, an antibody against PMCA1 (Swant, Switzerland) was used to show that the knockdown of PMCA4 was isoform specific as expression of PMCA1 was not altered by si-PMCA4 transfection (Fig, 3.3.1, A, middle panel). A western blot was also run to detect the levels of tubulin using an anti-tubulin

antibody (SIGMA-ALDRICH, UK) to determine equivalent loading of samples (Fig. 3.3.1, A, lower panel). Additionally, successful knockdown of PMCA4 by si-RNA transfection was also confirmed at the RNA level by real-time PCR analysis. PCRs performed using HUVEC transfected with si-PMCA4 and primers specific for PMCA4 (see appendix 3) had a reduction in PMCA4 RNA by 78% compared to si-NT transfected cells (Fig. 3.3.1, B). Furthermore, the knockdown of PMCA4 in endothelial cells by si-PMCA4 transfection was isoform specific as detection of PMCA1 by real-time PCR analysis using primers specific for PMCA1 (see appendix 3) resulted in no significant difference between the RNA levels of PMCA1 in si-NT or si-PMCA4 transfected cells (Fig. 3.3.1, C).

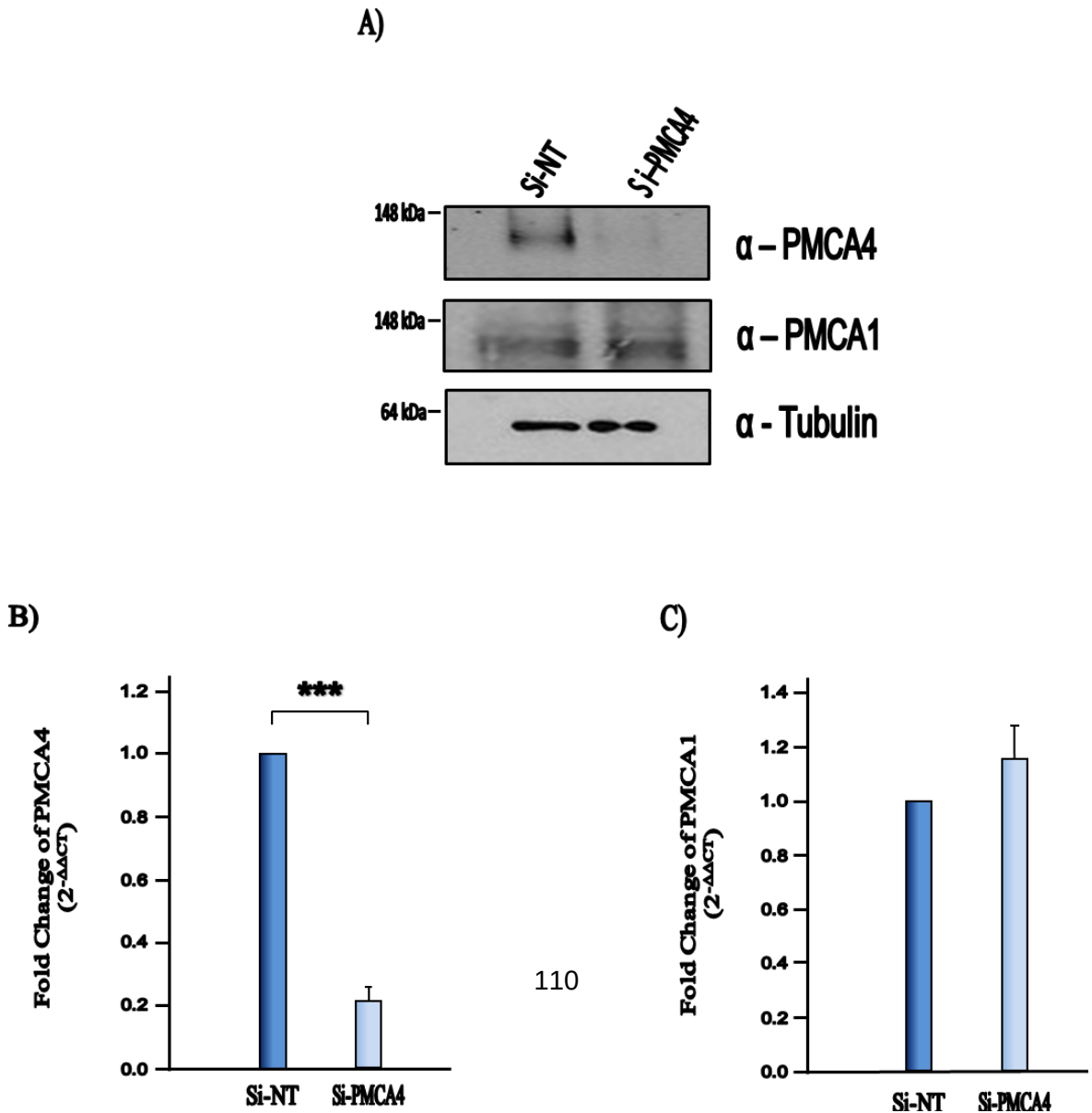


Fig. 3.3.1 Si-RNA-induced knockdown of PMCA4 at the protein and RNA level in HUVEC was successful A) Knockdown of PMCA4 by use of si-RNA in HUVEC. HUVEC were transfected with si-RNA specific for PMCA4 (si-PMCA4) or a control non-targeting si-RNA (si-NT) and protein expression was analysed by western blot using an antibody against PMCA4 (α-PMCA4), PMCA1 (α-PMCA1) or tubulin (α-tubulin). Transfection of HUVEC with si-PMCA4 resulted in a strong reduction in the protein expression of PMCA4 as compared to the levels observed in cells transfected with the si-NT control (A, upper panel). The expression of PMCA1 was unaffected by transfection with si-PMCA4 (A, middle panel) confirming specific knockdown of PMCA4. Equal amounts of protein were loaded in each case as equivalent bands were seen with both transfections after detection using an anti-tubulin antibody (A, lower panel). B) Successful knockdown of PMCA4 at the RNA level. HUVEC cells transfected with si-PMCA4 resulted in a decrease in PMCA4 RNA expression compared to si-NT (control) transfected cells when analysed by real-time PCR using primers specific for PMCA4. C) PCR analysis with primers specific for PMCA1 showed no significant difference between si-NT and si-PMCA4 transfected cells confirming isoform specific knockdown of PMCA4. The fold change for si-NT was set as 1 and si-PMCA4 values normalised to this as described in section 2.8.2. Data plotted as ± S.E. of three independent experiments, n=9 (*** $p \leq 0.001$).

3.3.2 Isolation of Mouse Lung Endothelial Cells Deficient in PMCA4

To demonstrate the successful knockout of PMCA4 in MLEC cells RNA was extracted from *PMCA4*$^{+/+}$ and *PMCA4*$^{-/-}$ MLEC, reverse transcribed into cDNA and used as a template for PCR amplification. PCR amplification using primers specific for PMCA4 (see appendix 2) produced a band of the expected size of 363bp (Fig. 3.3.2, upper panel) in PMCA4$^{+/+}$ endothelial cells that was absent in PMCA4$^{-/-}$ endothelial cells, confirming knockout of PMCA4 expression in MLEC. Additionally, primers detecting PMCA1 (see appendix 2) produced a band at the predicted size of 370bp (Fig. 3.3.2, middle panel) in samples from *PMCA4*$^{+/+}$ and *PMCA4*$^{-/-}$ mice demonstrating the RNA levels of PMCA1 are unaffected and therefore the knockout is isoform specific. To confirm equal amount of total cDNA in the PCR reactions from PMCA4$^{+/+}$ or PMCA4$^{-/-}$ samples, a control PCR of the housekeeping gene *Hprt1* was also performed. Agarose gel electrophoresis of the amplified fragment revealed equivalent amplification levels of a unique band of 85bp (Fig. 3.3.2, lower panel).

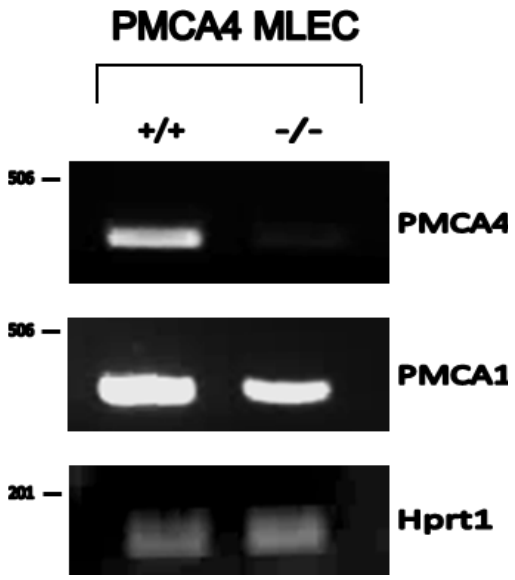


Fig. 3.3.2 Successful isolation of MLEC PMCA4$^{+/+}$ and PMCA4$^{-/-}$ cells. RNA was extracted from MLEC isolated from wild-type (*PMCA4*$^{+/+}$) or PMCA4 knockout (*PMCA4*$^{-/-}$) animals and reversed transcribed into cDNA. PCR amplification was then performed using primers specific for mouse PMCA4, PMCA1 and Hprt1 (control) and products analysed in 2% DNA agarose electrophoresis gels. Absence of PMCA4 amplification in the PCR reactions carried out with samples reversed-transcribed with RNA isolated from *PMCA4* $^{-/-}$ MLEC confirmed loss of PMCA4 expression in these cells

(upper panel). PCR reactions with oligonucleotides specific for PMCA1 showed that suppression of PMCA4 did not substantially affect the expression of PMCA1 (middle panel). Amplification with oligonucleotides specific for Hprt1confirmed equal amounts of cDNA in samples from wild-type or knockout MLEC (bottom panel).

3.3.3 Effect of Loss of PMCA4 on the Activity of the Calcineurin/NFAT Pathway

To evaluate the effect of PMCA4 suppression on the activity of the calcineurin/NFAT pathway, $PMCA4^{+/+}$ and $PMCA4^{-/-}$ MLEC were infected with Ad-NFAT-Luc and the calcineurin/NFAT signalling pathway activated by stimulation with PMA (20 ng/ml) and calcium Ionophore A23487 (1 µM) for 6 hours. Loss of PMCA4 resulted in a significant increase (325.18%, $p \leq 0.05$) in NFAT activity in the $PMCA4^{-/-}$ compared to the wild-type MLEC (Fig. 3.3.3) confirming that PMCA4 has a down-regulatory effect on NFAT activity and therefore is a negative-mediator of the calcineurin/NFAT pathway.

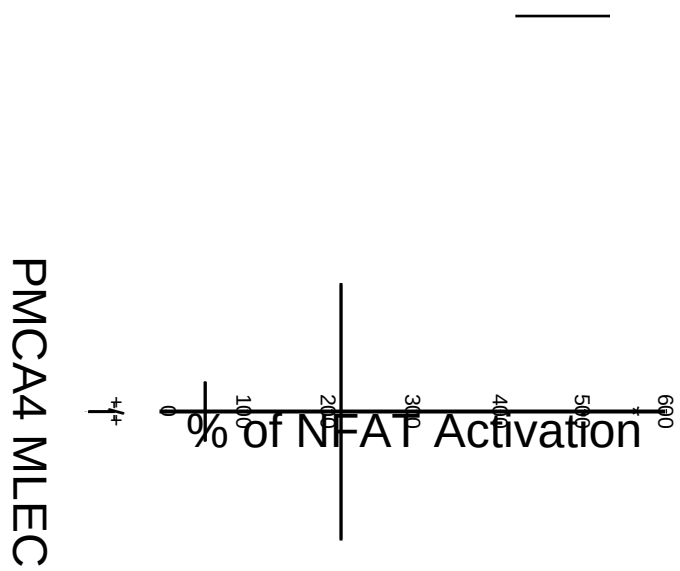


Fig. 3.3.3 Knockout of PMCA4 in MLEC results in an increase in NFAT activity. Loss of PMCA4 increases NFAT activity of MLEC in response to PMA and Ionophore stimulation. Wild-type ($PMCA4^{+/+}$) and PMCA4 knockout ($PMCA4^{-/-}$) MLEC were infected with Ad-NFAT-Luc and luciferase activity measured in a luminometer after 6 hour stimulation with PMA (20 ng/ml) and Ionophore (1 µM). Knockout of PMCA4 significantly increased NFAT activity compared to the activity in wild-type cells. Data plotted as mean ± S.E. of two independent experiments, n=5 (* $p \leq 0.05$).

3.4 Discussion

3.4.1 PMCA4 is a Negative-Regulator of the Calcineurin/NFAT Pathway in Endothelial Cells in Response to VEGF-Stimulation

PMCA4 has previously been reported to interact specifically with calcineurin in a variety of cells including; HEK293 (Buch *et al*, 2005), MCF-7 breast cancer cells (Holton *et al*, 2007), cardiomyocytes (Wu *et al*, 2009), PC12 cells (Kosiorek *et al*, 2011) and most interestingly, in the scope of this research, endothelial cells (Holton *et al*, 2010a). In HEK293 cells and neonatal rat cardiomyocytes PMCA4b down-regulates NFAT activation and has therefore been reported to act as an inhibitor of the calcineurin/NFAT pathway (Buch *et al*, 2005, Wu *et al*, 2009). This was also confirmed *in vivo* by localised over-expression of PMCA4b in the heart of mice. In response to TAC for two weeks, which induces pressure overload, NFAT activity was inhibited in the PMCA4b over-expressing heart compared to the control. Attenuation of cardiac hypertrophy in PMCA4b transgenic mice correlated with NFAT inhibition although a direct link with PMCA4b down-regulation of the calcineurin/NFAT pathway was not demonstrated. Interestingly, PMCA4b inhibition of cardiac hypertrophy only occurred in response to pathological but not physiological stimuli highlighting the potential of PMCA4b as a therapeutic target (Wu *et al*, 2009). In light of this data we focused our study specifically on the PMCA4 isoform although PMCA1

and 2 have also been shown to interact with calcineurin in endothelial cells (Holton *et al*, 2010a). PMCA1 would also be of interest to study as it is classed as the 'housekeeping' isoform (Okunade *et al*, 2004) and its expression, like PMCA4 is ubiquitous, whereas PMCA2 has a more tissue specific distribution localised to the brain, hair cells of the inner ear and the lactating mammary gland (Strehler and Zacharias, 2001). However, studies with PMCA1 pose potential challenges due to the knockout of this protein resulting in embryonic lethality (Okunade *et al*, 2004).

To evaluate the role of PMCA4 in the regulation of the calcineurin/NFAT pathway and angiogenesis we generated both 'gain of function' and 'loss of function' models. A successful gain of function model was produced by infection of HUVEC with an adenovirus that encodes PMCA4b resulting in specific ectopic expression of PMCA4. To further elucidate the regulatory role of PMCA4 two models for 'loss of function' were produced; PMCA4 knockdown, achieved by reducing PMCA4 expression in HUVEC using si-RNA and PMCA4 knockout, by isolating MLEC from *PMCA4*$^{-/-}$ mice. Furthermore, VEGF-A_{165} was selected as the appropriate stimulus in this study as it is the predominant isoform (Liekens *et al*, 2001) and has been shown in HUVEC to increase NFAT activity which is dependent on calcineurin activation (Armesilla *et al*, 1999). Our data confirms the previously reported inhibitory function of PMCA4 on NFAT activity (Buch *et al*, 2005; Wu *et al*, 2009) also occurs in endothelial cells as over-expression of PMCA4 attenuated VEGF-induced NFAT activation identifying PMCA4 as a negative-regulator of the calcineurin/NFAT pathway, specifically in endothelial cells. In agreement with this, a 'loss of function' study using the PMCA4 knockout model resulted in a significant increase in NFAT

activity in the PMCA4$^{-/-}$ cells compared to the wild-type (PMCA4$^{+/+}$) in response to PMA and ionophore stimulation. This is also in accordance with the findings of Wu *et al*, 2009 who used a si-RNA method of PMCA4b knockdown in rat cardiomyocytes which resulted in an up-regulation of NFAT activity in response to phenylephrine-stimulation compared to the control (Wu *et al*, 2009).

3.5 Conclusion

In conclusion, we confirmed successful over-expression, knockdown and knockout models of PMCA4 in endothelial cells for use in 'gain of function' and 'loss of function' studies to elucidate the involvement of PMCA4 in calcineurin/NFAT signalling and angiogenesis. Additionally, this data has shown ectopic expression of PMCA4 down-regulates NFAT activity which was confirmed in the PMCA4 knockdown model establishing PMCA4 as a negative-regulator of the calcineurin/NFAT pathway in response to VEGF-stimulation in endothelial cells. This highlights the potential of PMCA4 as an endogenous regulator of this signalling pathway in endothelial cells in a variety of physiological and pathological settings, such as angiogenesis, where this pathway has been established to be involved (Hernández *et al*, 2001).

4. CHAPTER FOUR

Results

Effect of PMCA4 on the Regulation of Angiogenesis

4.1 Introduction

Successful angiogenesis requires several events including basement membrane degradation, cell migration, proliferation and tube formation (Liekens *et al*, 2001). A number of signalling pathways regulated by the pro-angiogenic factor, VEGF, are implicated in processes required for angiogenesis such as; p38 MAPK (McMullen *et al*, 2005), PI3K (Fujio and Walsh, 1999) and of particular interest, PLC-γ (Holmes *et al*, 2010) which activates the calcineurin/NFAT pathway (Crabtree and Olson, 2002). Inhibition of calcineurin by CsA has been shown to inhibit angiogenesis (Hernández *et al*, 2001), however, use of CsA is associated with severe side effects (Matrínez-Martínez and Redondo, 2004) and it would be beneficial to find an endogenous regulator of this pathway that could be a potential therapeutic target in the future. We hypothesise this molecule could be PMCA as it has previously been reported that PMCA4 and PMCA2 are negative-regulators of the calcineurin/NFAT pathway in HEK293 cells (Buch *et al*, 2005; Holton *et al*, 2007). Our data suggests down-regulation of the activity of the calcineurin/NFAT pathway by PMCA4 also occurs in endothelial cells (see chapter 3). We therefore hypothesise that over-expression of PMCA4 will result in inhibition of angiogenic processes regulated by the calcineurin/NFAT pathway. To evaluate this

hypothesis we have performed PMCA4 ‘gain’ and ‘loss’ of function experiments in endothelial cells and analyzed the effect of PMCA4 excess or deficiency on the expression of NFAT-dependent, pro-angiogenic genes and on endothelial cell migration, tubular morphogenesis (*in vitro* and *in vivo*) and proliferation. The results of these experiments are described in the following sections.

4.2 PMCA4 Inhibits VEGF-Induced Expression of the Pro-Angiogenic Proteins RCAN1.4 and Cox-2 at Both the Protein and mRNA Levels

RCAN1.4 and Cox-2 have been implicated in processes required for successful angiogenesis such as cell migration (Iizuka *et al*, 2004; Holmes *et al*, 2010; Hernández *et al*, 2001) and tube formation (Iizuka *et al*, 2004; Hernández *et al*, 2001; Woods *et al*, 2003). As their expression is regulated by the VEGF-mediated activation of the calcineurin/NFAT pathway (Iizuka *et al*, 2004; Hernández *et al*, 2001) these genes were of particular interest as they could potentially be regulated by PMCA4. To test this possibility HUVEC were infected with adenovirus encoding PMCA4 or LacZ as previously described. Cells were serum-starved in 0.5% fetal calf serum and then stimulated with VEGF (25 ng/ml) for 4 hours. Protein lysates of infected cells were analysed by western blot using antibodies against RCAN1.4 (SIGMA-ALDRICH, UK), Cox-2 (Alexis® Biochemicals, UK) and Cox-1 (Alexis® Biochemicals, UK) (see appendix 1 and 6 for table of antibodies and all western blot images respectively). Over-expression of PMCA4 resulted in a significant decrease in RCAN1.4 and Cox-2 protein expression by 48.42% and 52.1%, $p \leq 0.001$, respectively compared to the control, whereas Cox-1 levels remained unaffected (Fig. 4.2.1). A western blot detecting tubulin was carried out to confirm equal loading of all samples (Fig. 4.2.1).

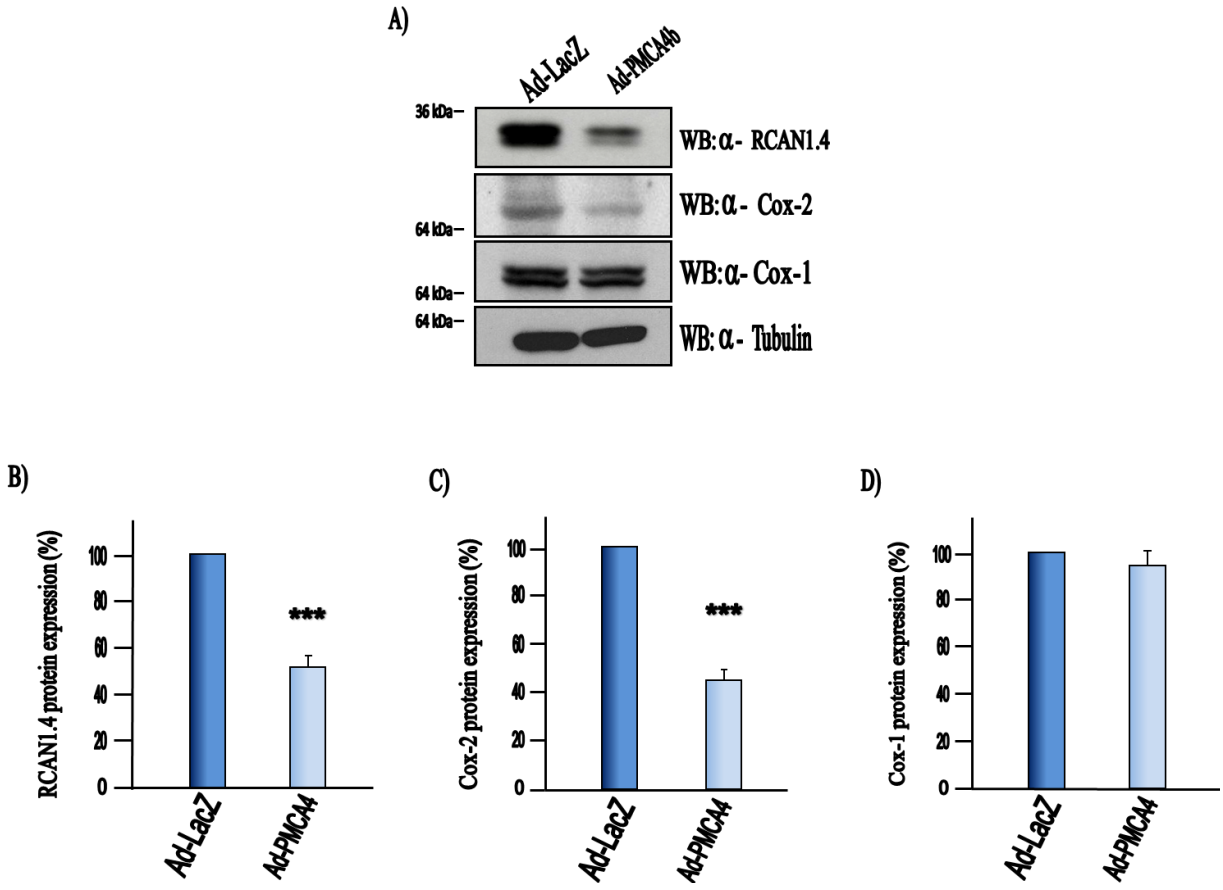


Fig. 4.2.1 Ectopic expression of PMCA4b attenuates the protein expression levels of RCAN1.4 and Cox-2 in HUVEC cells stimulated with VEGF. HUVEC were infected for 48 hours with either Ad-LacZ or Ad-PMCA4b. After serum starvation cells were stimulated with VEGF (25 ng/ml) for 4 hours and protein expression was analysed by western blot. A) Representative western blot images of protein expression (see appendix 6 for all images). Ectopic expression of PMCA4b reduces the expression levels of RCAN1.4 (WB:α-RCAN1.4) and Cox-2 (WB:α-Cox-2) in endothelial cells. The levels of Cox-1 (WB:α-Cox-1) remain unaffected and the loading control tubulin (WB:α-Tubulin) was equivalent in all cases. B, C, D) Histograms showing analysis of western blot experiments. The density of bands from western blot experiments was analysed

using Image J software (as stated in section 2.8.1) and the results represented in histograms that show data as mean ± S.E. of two independent experiments, n=5 (*** $p \leq 0.001$).

RNA levels for RCAN1.4 and Cox-2 were also determined by real-time PCR analysis using specific primers for each gene (see appendix 3). Both RCAN1.4 and Cox-2 expression at the RNA level was also found to be reduced in the presence of ectopic PMCA4 by 28.84% and 29.44% respectively in HUVEC infected cells stimulated with VEGF (25 ng/ml) for 2 hours (Fig. 4.2.2)

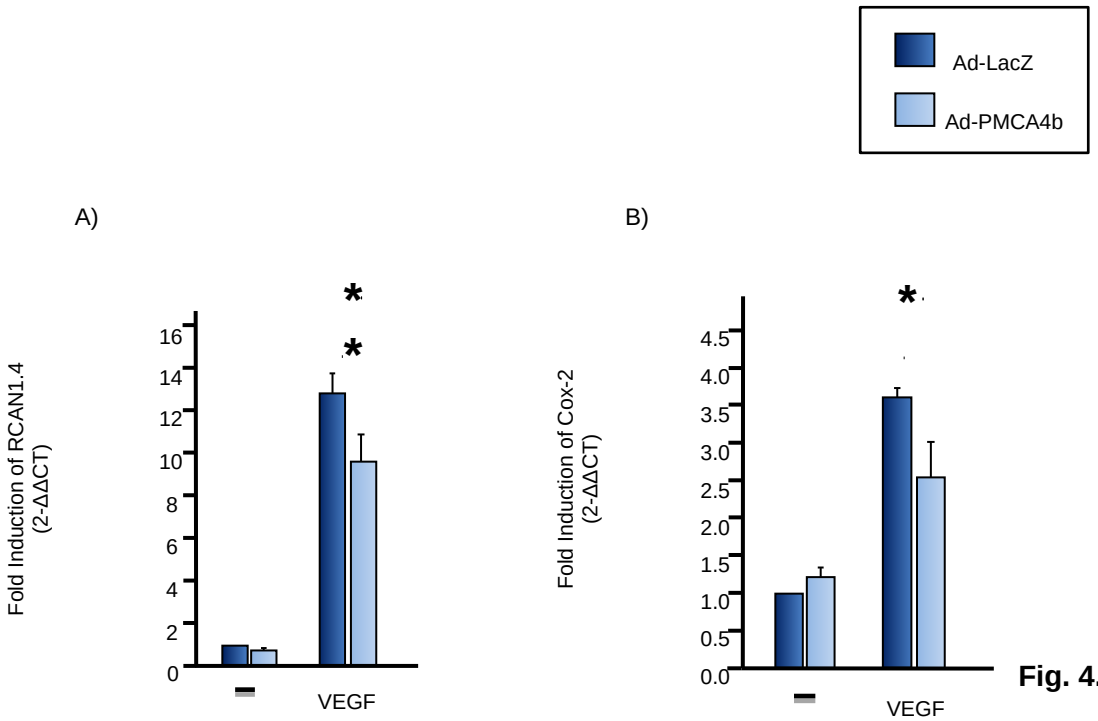


Fig. 4.2.2 Ectopic expression of PMCA4b significantly reduces the fold induction of the pro-angiogenic genes *RCAN1.4* and *Cox-2*. HUVEC cells were infected with Ad-LacZ (control) or Ad-PMCA4b. After serum starvation overnight, cells were stimulated with VEGF (25 ng/ml) for two hours and RNA extracted. The RNA levels encoding RCAN1.4 and Cox-2 were analysed by quantitative real-time PCR using oligonucleotides specific for each gene (see appendix 3). Results were analysed using the $2^{-\Delta\Delta CT}$ method (see section 2.8.2). Ad-LacZ unstimulated samples were set as 1 and the fold induction for the other conditions calculated relative to this. A) Expression of PMCA4b significantly reduced the up-regulation of RCAN1.4 after stimulation with VEGF. Mean ± S.E. of two

independent experiments, n=6 (** $p \leq 0.01$) is shown. B) The VEGF-mediated increment in Cox-2 RNA levels was also decreased by over-expression of PMCA4b. Mean ± S.E of two independent experiments, n=5 (* $p \leq 0.05$) is shown.

These results demonstrate that PMCA4 over-expression down-regulates the expression of the NFAT dependent genes *RCAN1.4* and *Cox-2* but does not exert any influences on the expression of Cox-1 (which is not regulated via calcineurin/NFAT) suggesting that PMCA4 attenuates the expression of the pro-angiogenic genes *RCAN1.4* and *Cox-2* via inhibition of the calcineurin/NFAT pathway.

In support of this data the knockdown of PMCA4 conversely yielded an increase in RCAN1.4 protein expression. HUVEC were transfected with si-RNA corresponding to PMCA4 (si-PMCA4) or the control non-target (si-NT) and stimulated with VEGF as above. Silencing of PMCA4 resulted in a significant increase in the protein expression of RCAN1.4 compared to the control (Fig.4.2.3, A). Interestingly, knockdown of PMCA4 increased RCAN1.4 protein expression both at the basal level and in response to VEGF-stimulation with an up-regulation of 91% and 29.97% respectively compared to the control (si-NT) (Fig.4.2.3, B).

Similar results were observed in western blot analysis of $PMCA4^{+/+}$ and $PMCA4^{-/-}$ MLEC after 4 hour stimulation with VEGF (50 ng/ml) using an antibody against RCAN1.4 (Fig. 4.2.4) although this variable was not quantified by densitometry. In both cases western blots detecting tubulin were carried out to ensure equal loading of all samples (Fig. 4.2.3 and Fig. 4.4.4).

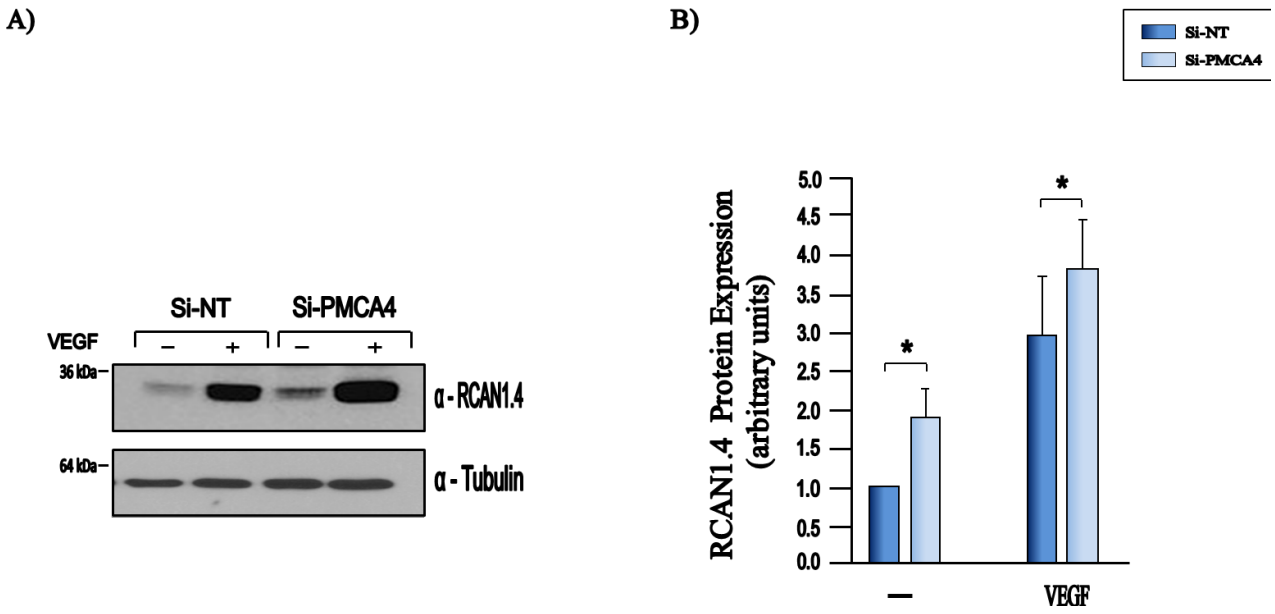


Fig.4.2.3 Knockdown of PMCA4b in HUVEC significantly increases RCAN1.4 protein expression both at the basal level and in response to VEGF-stimulation. HUVEC were transfected with si-RNA corresponding to PMCA4 (si-PMCA4) or the control non-target (si-NT), stimulated with VEGF (25 ng/ml) for 4 hours and analysed by western blot. A) Representative western blot images (see appendix 7 for all images) showing that loss of PMCA4 increases the protein expression of RCAN1.4 (α-RCAN1.4). A western blot detecting tubulin (α-Tubulin) confirmed equal amounts of protein in the samples. B) Histogram shows analysis of western blot images using Image J software. Knockdown of PMCA4 increases RCAN1.4 protein expression both in the absence and presence of the VEGF-stimulus. Data plotted as mean ± S.E. of two independent experiments, n=3 (* $p \leq 0.05$).

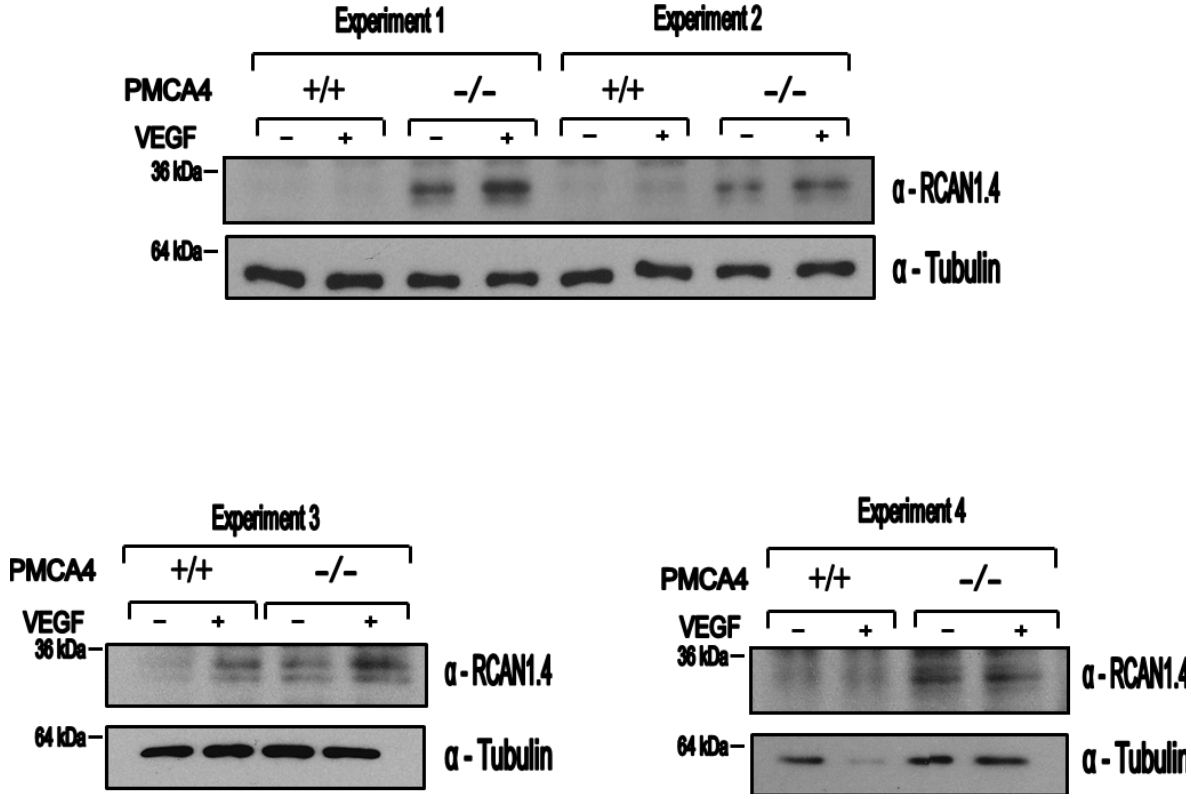


Fig. 4.2.4 Knockout of PMCA4 in MLEC increases RCAN1.4 protein expression in response to VEGF-stimulation. Isolated wild-type MLEC (PMCA4$^{+/+}$) and PMCA4 knockout (PMCA4$^{-/-}$) were stimulated with VEGF (50 ng/ml) after serum starvation and analysed by western blot using an antibody against RCAN1.4 (α-RCAN1.4). In all cases a western blot detecting tubulin (α-tubulin) was carried out to check protein concentration in different samples. PMCA4$^{-/-}$ resulted in an increase in the VEGF-induced protein expression of RCAN1.4 compared to PMCA4$^{+/+}$ in three independent experiments, n=4.

4.3 VEGF-Induced Endothelial Cell Migration is Inhibited in the Presence of Ectopic PMCA4b

As we had shown PMCA4 was a negative regulator of the pro-angiogenic factors RCAN1.4 and Cox-2 we wanted to determine if this had a knock on effect on the processes they have been reported to be involved in such as cell migration (Iizuka *et al*, 2004; Holmes *et al*, 2010; Hernández *et al*, 2001). HUVEC cells were infected with adenovirus as previously mentioned and a 0.9 mm gap created using a gap generator from a wound healing assay kit (Cell Biolabs Inc, UK). At time zero and after 24 hours of incubation with VEGF (25 ng/ml) cells were stained and fixed. Endothelial cell migration was found to be significantly reduced (39.80% reduction, $p \leq 0.05$) by ectopic expression of PMCA4b compared to the migration of control cells infected with Ad-LacZ (Fig. 4.3.1), highlighting PMCA4s role as a negative-regulator of endothelial cell migration in response to VEGF.

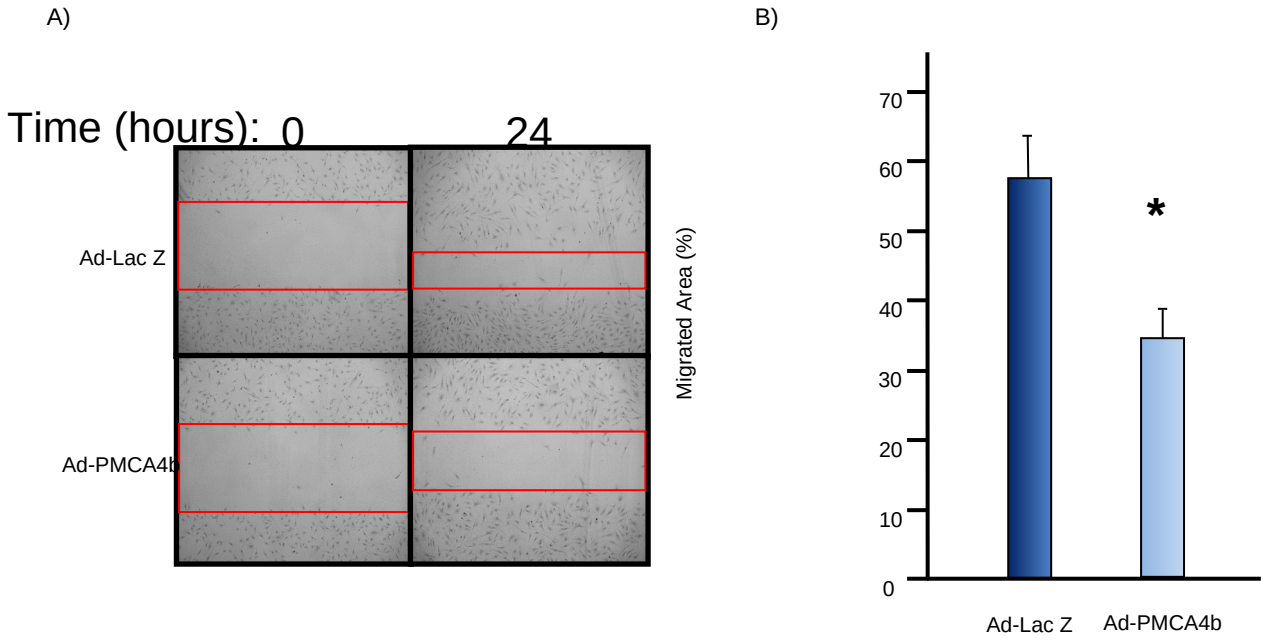


Fig. 4.3.1 Ectopic expression of PMCA4b prevents cell migration of endothelial cells stimulated with VEGF. Infected HUVEC (Ad-LacZ or Ad-PMCA4b) were fixed at time zero or after incubation in ECGM supplemented with VEGF (25 ng/ml) for 24 hours. (A) VEGF-induced endothelial cell migration was significantly attenuated with ectopic expression of PMCA4b. Images are representative of endothelial cell migration assays after HUVEC infection with Ad-LacZ or Ad-PMCA4. B) The amount of migration was calculated from all images (see appendix 8 for all images used in quantification) by calculating the unmigrated area (the area shown by red boxes that contain no cells) as a percentage in relation to the value for the total area in the corresponding time zero image (set as 100%) (again represented by red boxes). Subtraction of the unmigrated value from 100% gave the percentage of migration. Data plotted as mean ± S.E. of three independent experiments, n=6 (* $p \leq 0.05$).

To substantiate this observation, we studied the effect of PMCA4 knockout on endothelial cell migration. MLEC PMCA4$^{+/+}$ and PMCA4$^{-/-}$ cells were plated as above and analysed at time zero and after 24 hours of stimulation with VEGF (50 ng/ml). PMCA4 deletion resulted in a significant increase in VEGF-induced endothelial cell migration by 66.62%, $p \leq 0.01$, compared to the migration of wild-type MLEC (PMCA4$^{+/+}$) (Fig. 4.3.2) reassuring our hypothesis of PMCA4 function as a down-regulator of endothelial cell migration, one of the essential processes required for successful angiogenesis.

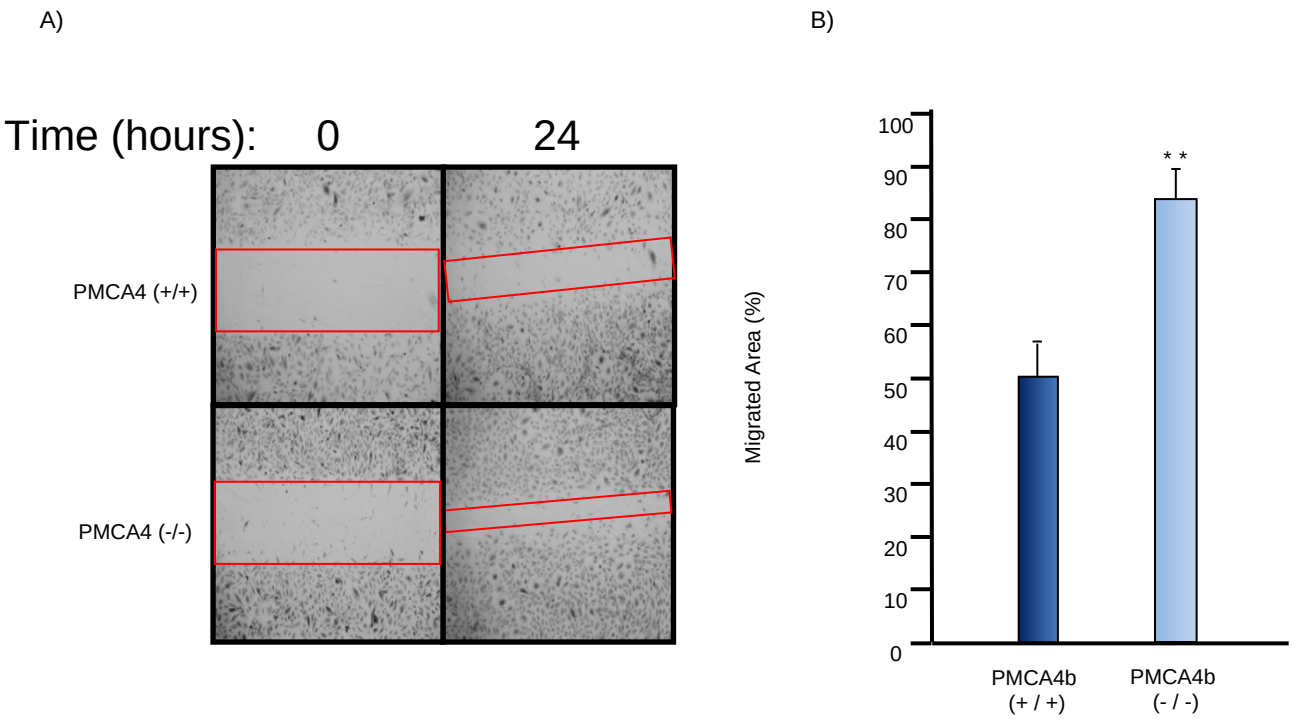


Fig. 4.3.2 Knockout of PMCA4 augments VEGF-induced endothelial cell migration. Isolated MLEC from wild-type (*PMCA4*$^{+/+}$) and PMCA4 knockout (*PMCA4*$^{-/-}$) mice were plated in the presence of a gap creator which was subsequently removed allowing cell migration into the wound. Migration was measured at time zero and after 24 hours of incubation of cells in MLEC growth medium complete supplemented with VEGF (50 ng/ml). A) Images were used to calculate the migrated area as mentioned in the over-expression study (see appendix 9 for all images). B) Loss of PMCA4 in PMCA4$^{-/-}$ endothelial cells resulted in a significant increase in cell migration in response to VEGF-stimulation compared to that in the wild-type cells PMCA4$^{+/+}$. Data plotted as mean ± S.E. of two independent experiments n=4 and n=6 for PMCA4$^{+/+}$ and PMCA4$^{-/-}$ respectively (** $p \leq 0.01$).

4.4 *In Vitro* Tube Formation in Response to VEGF-Stimulation is Attenuated With Over-Expression of PMCA4b

Tube formation is another process required for successful angiogenesis (Carmeliet, 2000). RCAN1.4 and Cox-2 have been implicated in the stimulation of tube formation (Iizuka *et al*, 2004; Hernández *et al*, 2001; Woods *et al*, 2003) therefore we decided next to investigate the role of PMCA4 as a regulator of tubular morphogenesis by endothelial cells. To study the effect of PMCA4 over-expression on tube formation, HUVEC cells were infected with Ad-LacZ or Ad-PMCA4b and plated onto a layer of growth-factor reduced matrigel. Cells were then incubated in medium 200 including 2% FBS or 2% FBS with additional VEGF (50 ng/ml) for 24 hours. After this time cells were fixed and images taken by microscopic examination (see appendix 10 for full set of images). The number of tubes in unstimulated cells infected with Ad-LacZ were taken as a reference and assigned a value of 100%. Tube formation in the other conditions was calculated as a percentage relative to this control. Interestingly, ectopic expression of PMCA4b attenuated tube formation both in response to VEGF and also in the absence of the stimulus by 49.01% and 34.29% respectively (Fig. 4.4.1). These results suggest that PMCA4 is a negative-regulator of endothelial tube formation.

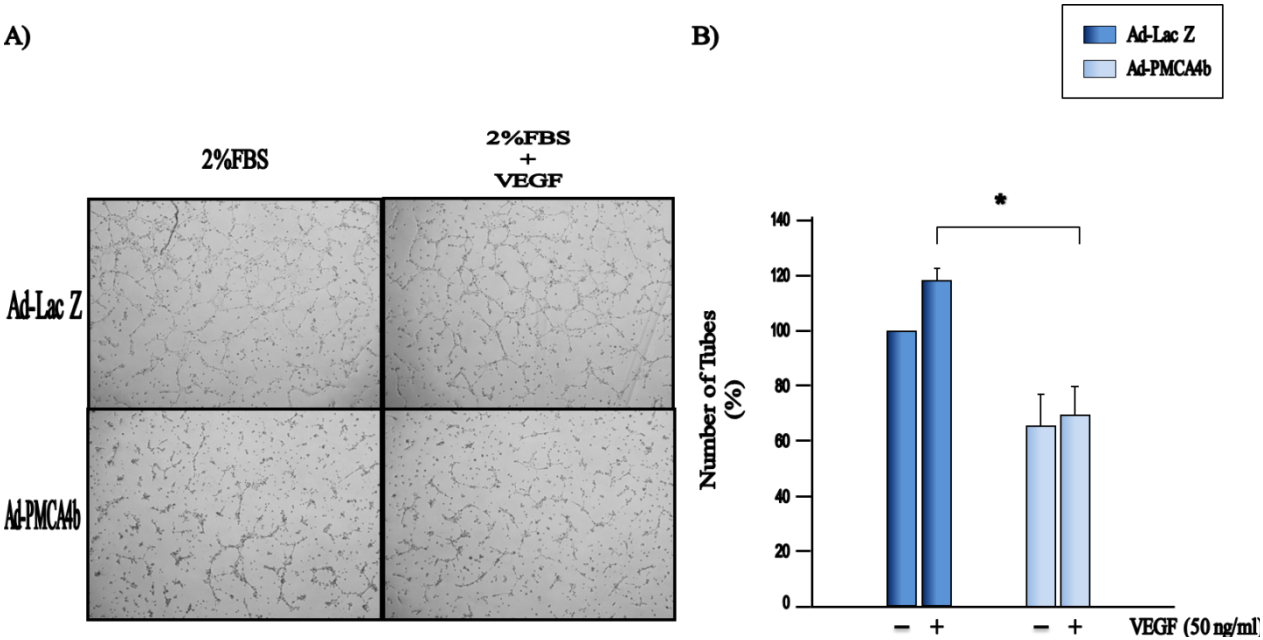


Fig. 4.4.1 *In vitro* tube formation of endothelial cells in response to VEGF-stimulation is reduced by over-expression of PMCA4b. Infected HUVEC (Ad-LacZ or Ad-PMCA4b) were incubated with 2% FBS (control) (-) or 2% FBS with additional VEGF (50 ng/ml) for 24 hours (+). (A) A representation of images used for the calculation of the percentage of tube formation (see appendix 10 for all images). (B) Ectopic expression of PMCA4b significantly reduced both the basal and VEGF-induced tube formation of endothelial cells. Data plotted as mean ± S.E. of two independent experiments, n=at least five (* $p \leq 0.05$).

To confirm this observation we analysed the effect of PMCA4 knockdown on endothelial cell tube formation. HUVEC cells were transfected with si-PMCA4 or the non-target control (si-NT) as previously mentioned. Cells were plated onto matrigel and incubated in 2% FBS (control) or 2% FBS supplemented with

VEGF (50 ng/ml) for 24 hours. Loss of PMCA4 significantly increased tube formation by HUVEC in the absence and presence of VEGF compared to the control by 23.07% and 22.05%, $p \leq 0.01$, respectively (Fig. 4.4.2), reassuring the role of PMCA4 as a down-regulator of tube formation.

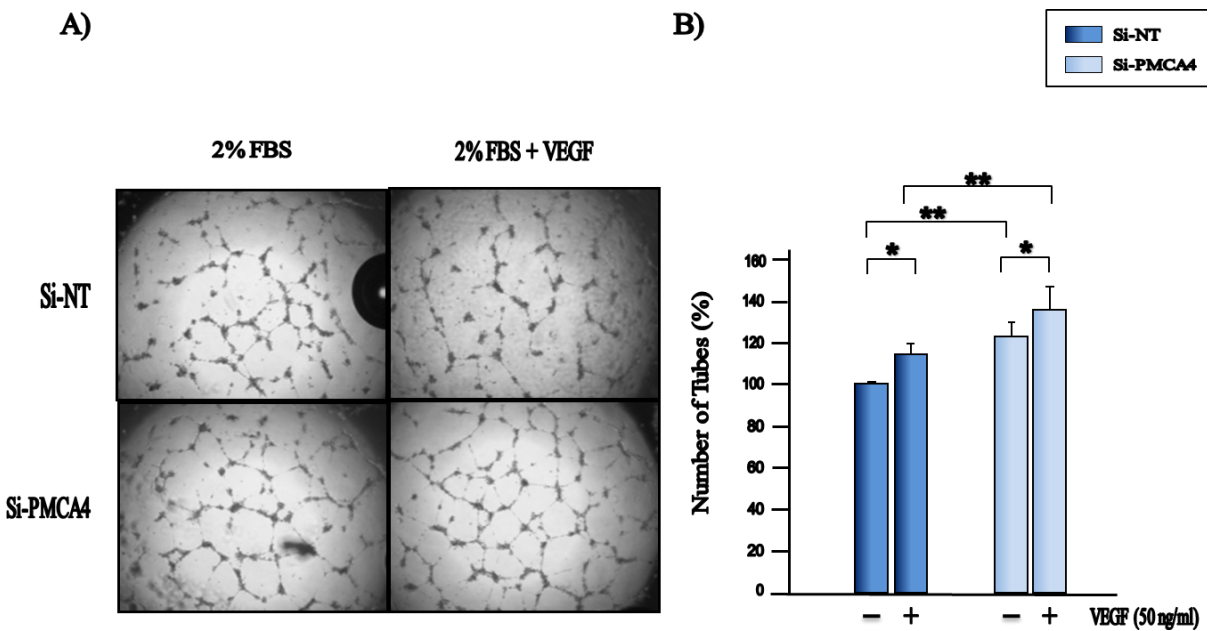


Fig. 4.4.2 Knockdown of PMCA4 significantly increases tube formation of endothelial cells *in vitro* in both the absence and presence of VEGF. HUVEC transfected with the control non-target (si-NT) or si-RNA corresponding to PMCA4 (si-PMCA4) were plated onto growth factor-reduced matrigel and stimulated with 2% FBS (control) (-) or 2% FBS supplemented with VEGF (50 ng/ml) for 24 hours (+). (A) A representation of images used for the calculation of the percentage of tube formation. Non-target (si-NT) was set as 100% and the percentage of tube formation in the other conditions calculated from this value (see appendix 11 for all images). (B) Loss of PMCA4 significantly increased tube formation at the basal level and in response to VEGF-stimulation. Histogram represents mean ± S.E. of two independent experiments, n=7 (* $p \leq 0.05$) (** $p \leq 0.01$).

4.5 *In Vivo* Over-Expression of PMCA4b Down-Regulates the Formation of Functional Vessels in Response to VEGF-Stimulation

We are collaborating in this project with Professor Redondo's research group (at the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares CNIC, Madrid, Spain) to analyse the effect of

PMCA4 over-expression in an *in vivo* model of angiogenesis. Professor Redondo's group implanted a mixture of growth factor-reduced matrigel containing VEGF (500 ng/ml), heparin (376 µg/ml) and $2x10^9$ pfu of the corresponding adenovirus (Ad-LacZ or Ad-PMCA4), subcutaneously in 4 week old anesthetised mice. Seven days later the plugs were removed and half was used to measure haemoglobin content, by colorimetric assay at 540nm, and the other half embedded in paraffin, sliced and stained with haematoxylin and eosin to detect the number of vessel like structures containing red blood cells. Over-expression of PMCA4 significantly reduced both the haemoglobin content and the number of vessels containing blood by 47.1% and 58.19%, $p \leq 0.05$, respectively compared to the control (Fig. 4.5.1, B and C). Professor Redondo has very kindly granted permission to present these results in this thesis.

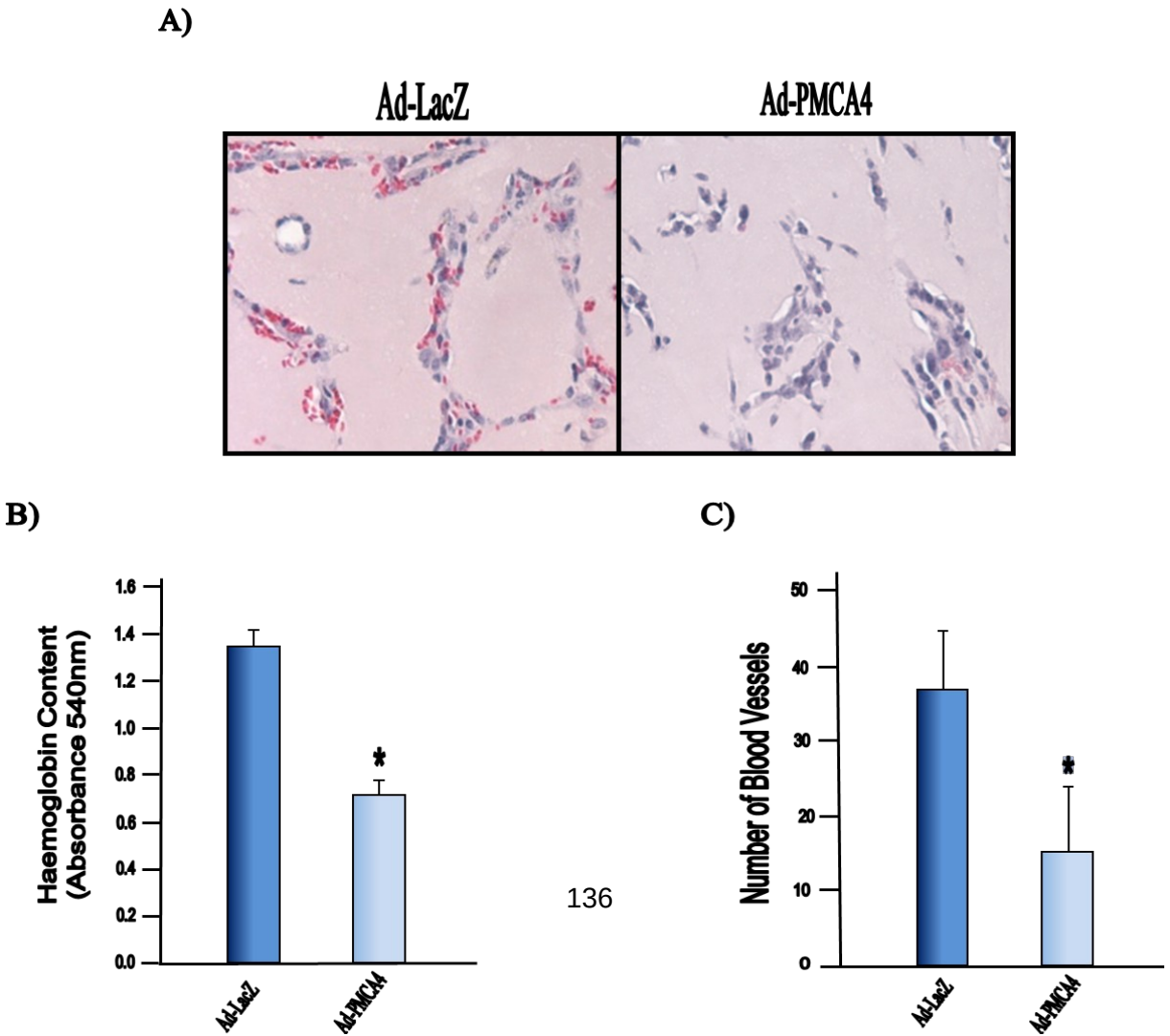


Fig. 4.5.1 VEGF-induced tube formation *in vivo* is inhibited with ectopic expression of PMCA4b. Matrigel plugs containing VEGF and Ad-LacZ or Ad-PMCA4 were injected subcutaneously into mice. One week later plugs were analysed for haemoglobin content and the number of blood vessels. The matrigel plug containing PMCA4 had significantly fewer functional vessels than the control. A) Plugs were stained with haematoxylin to detect endothelial cells (blue) or eosin to identify red blood cells (pink). Plugs formed in the presence of Ad-LacZ present numerous vessel-like structures containing red blood cells. In comparison, in plugs containing Ad-PMCA4 most of the vessel like structures appear empty. B) The haemoglobin content measured at 540nm wavelength is significantly reduced in the matrigel plugs containing PMCA4. Data plotted as mean ± S.E. of three independent experiments with at least seven animals per group in each experiment (* $p \leq 0.05$). C) The number of blood vessels was also significantly attenuated in PMCA4 containing plugs. Histogram represents mean ± S.E. of three independent experiments (* $p \leq 0.05$).

4.6 Over-Expression of PMCA4b has no Effect on VEGF-Induced Proliferation of Endothelial Cells or the Activation of Erk1/2 MAPK

Proliferation is another process required for angiogenesis (Liekens *et al*, 2001). To study the effect of PMCA4 over-expression on endothelial cell proliferation HUVEC were infected with Ad-LacZ or Ad-PMCA4b and proliferation measured by standard MTT assay after 3 and 6 days of incubation in ECGM complete supplemented with additional VEGF (25 ng/ml). Ectopic expression of PMCA4b

had no significant effect on endothelial cell proliferation compared to the proliferation in control cells infected with Ad-LacZ, suggesting that PMCA4 is not a regulator of this process (Fig. 4.6.1).

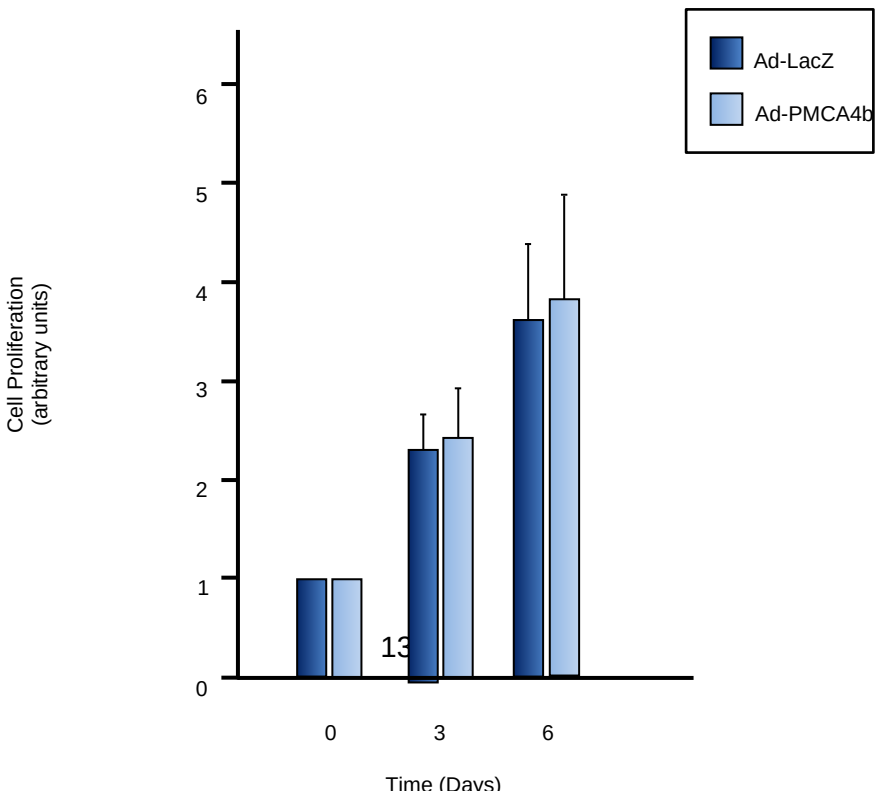


Fig. 4.6.1 Ectopic expression of PMCA4b has no effect on endothelial cell proliferation. Endothelial cell proliferation for cells infected with Ad-LacZ (control) or Ad-PMCA4b were measured by standard MTT assay at time zero and after three and six days of incubation in ECGM complete supplemented with 25 ng/ml VEGF. Over-expression of PMCA4b resulted in no significant differences in cell proliferation compared to the control. Histogram shows data as mean ± S.E. of three independent experiments, n=6.

To confirm the above result the phosphorylation (activation) status of Erk1/2 MAPK was analysed as it has been reported that Erk1/2 are implicated in VEGF-induced cell proliferation of HUVEC (Yu and Sato, 1999). HUVEC cells were infected as above and left unstimulated or stimulated with VEGF (25 ng/ml) for 5 minutes. Western blot analysis using an antibody against phosphorylated Erk (Cell Signalling, UK) showed no significant difference in the

phosphorylation status, and therefore activation of Erk, in cells over-expressing PMCA4b or control cells infected with Ad-LacZ. Total levels of Erk were also detected using an antibody against total Erk (Cell Signalling, UK) to normalise the phosphorylated values (Fig. 4.6.2).

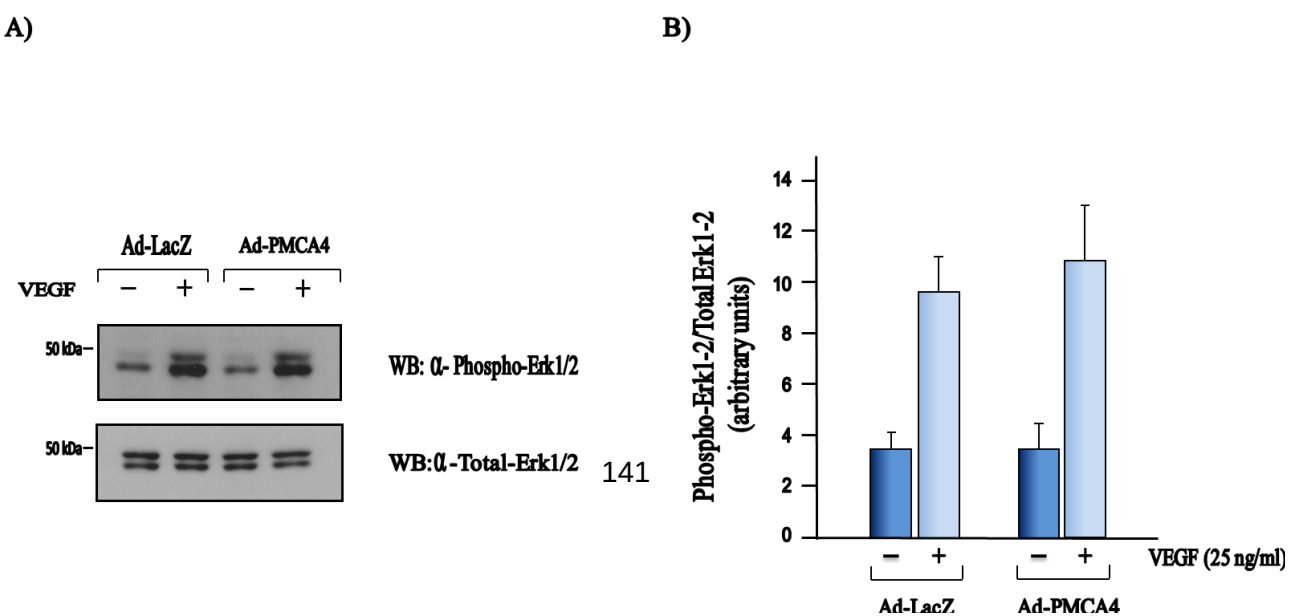
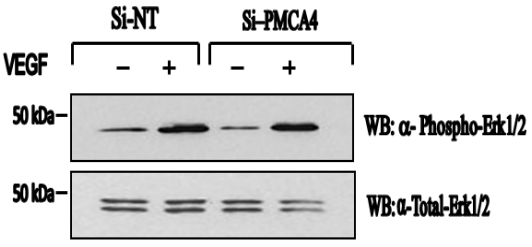


Fig. 4.6.2 VEGF-induced phosphorylation of endothelial Erk1/2 is unaffected by ectopic expression of PMCA4b. Endothelial cells infected with Ad-LacZ or Ad-PMCA4b were left unstimulated (-) or stimulated (+) with VEGF (25 ng/ml) for 5 minutes. (A) Western blots detecting phosphorylated Erk1/2 (WB:α-Phospho-Erk1/2) and total Erk1/2 (WB:α-Total-Erk1/2) showed no significant difference between control cells or those over-expressing PMCA4 (see appendix 12 for all images). (B) Histogram shows data as mean ± S.E. of two independent experiments, n=6. In each case the density of the bands for phosphorylated Erk1/2 were normalised to the density of the corresponding band for total Erk1/2.

This result was further supported by observing the effect of knockdown of PMCA4 on the phosphorylation of Erk1/2. HUVEC were transfected with si-RNA specific for PMCA4 (si-PMCA4) or non-target (si-NT) and treated as above. PMCA4 knockdown or over-expression yielded similar results, in that no significant difference in the phosphorylation and therefore activation of Erk1/2 occurred (Fig. 4.6.3). Altogether, these data indicate that PMCA4 is not a regulator of endothelial cell proliferation in response to VEGF-stimulation.

A)


Si-NT
Si-PMCA4
VEGF
– + – +
50 kDa
WB: α- Phospho-Erk1/2
50 kDa
WB: α-Total-Erk1/2

B)

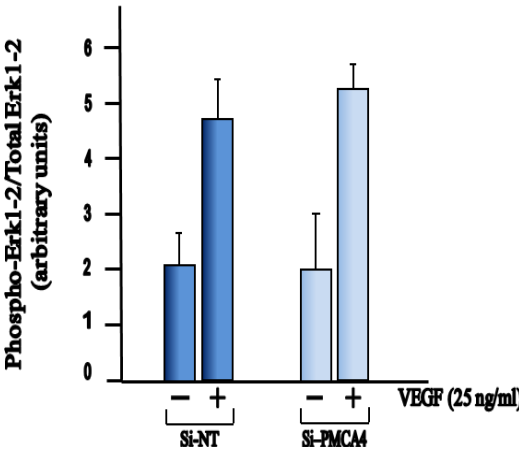

Phospho-Erk1-2/Total Erk1-2
(arbitrary units)
0
1
2
3
4
5
6
– + – +
VEGF (25 ng/ml)
Si-NT
Si-PMCA4

Fig. 4.6.3 Knockdown of PMCA4 has no significant effect on the phosphorylation of Erk1/2 in response to VEGF-stimulation. HUVEC cells were transfected with si-RNA specific for PMCA4 or non-targeting control and stimulated with VEGF (25 ng/ml) for 5 minutes (+) or left unstimulated (-). A) Samples were analysed by western blot using an antibody against phosphorylated Erk (WB:α- Phospho-Erk1/2) showing no significant difference between the phosphorylation (activation) of Erk1/2 in control cells or those where PMCA4 was silenced. A western blot detecting total Erk1/2 (WB:α-Total-Erk1/2) was also performed as a control (see appendix 13 for all images used in quantification). (B) The density of bands for phosphorylated Erk1/2 were normalised to the value obtained for total Erk1/2 (α-Total Erk1/2) and plotted as mean ± S.E. of two independent experiments, n=3.

4.7 Discussion

As angiogenesis is associated with over 70 diseases it highlights an important aspect of disease progression and therefore regulation of angiogenesis by therapeutic targeting would be advantageous in a plethora of illnesses (Carmeliet, 2005). Successful angiogenesis requires several events such as, proliferation, cell migration and tube formation (Carmeliet, 2000). Numerous pathways are involved in the regulation of these processes but of particular interest is the calcineurin/NFAT signalling pathway which has been shown to

regulate a number of these events (Hernández *et al*, 2001). We identified PMCA4 as an inhibitor of this pathway in endothelial cells (see chapter 3) and therefore aimed to establish if PMCA4 could also regulate calcineurin/NFAT dependent angiogenic processes.

4.7.1 The Protein Expression and mRNA Levels of the Pro-Angiogenic Factors RCAN1.4 and Cox-2 are Negatively-Regulated by PMCA4

It has previously been reported that the expression and activation of RCAN1.4 and Cox-2 in response to VEGF-stimulation is regulated by the calcineurin/NFAT pathway and both have been shown to be pro-angiogenic proteins by regulating processes required for angiogenesis such as cell migration and tube formation (Iizuka *et al*, 2004; Hernández *et al*, 2001). As we have previously demonstrated that PMCA4 is a negative-regulator of the calcineurin/NFAT pathway in endothelial cells we wanted to identify if PMCA4 could also regulate these NFAT-dependent proteins.

RCAN1 RNA levels have been shown to increase in HUVEC in response to VEGF-signalling (Hesser *et al*, 2004). Specifically, RCAN1.4 mRNA is elevated in response to VEGF-stimulation in endothelial cells, such as HUVEC, in a calcineurin-dependent mechanism, whereas RCAN1.1 levels remain the same (Yao and Duh, 2004; Holmes *et al*, 2010; Minami *et al*, 2004). Additionally, the presence of RCAN1.4, but no other isoforms, was identified in VEGF-induced HUVEC (Iizuka *et al*, 2004). The conditions used in our study, such as $VEGF_{165}$-stimulation and HUVEC, suggest we are studying in particular the RCAN1.4 isoform.

We found ectopic expression of PMCA4b in VEGF-induced HUVEC significantly attenuated both the protein and RNA levels of RCAN1.4. In support of this finding, knockdown of PMCA4 significantly increased the protein expression of RCAN1.4 both in the absence and presence of VEGF compared to the control. Similarly to RCAN1.4, Cox-2 protein and mRNA expression has been reported to be increased in HUVEC in response to VEGF-stimulation and is inhibited by CsA, showing a calcineurin-dependent mechanism of expression (Hernández *et al*, 2001). We also established ectopic expression of PMCA4 significantly inhibited the protein and mRNA levels of Cox-2 in VEGF-induced HUVEC compared to the control. This data demonstrates PMCA4 is a negative-regulator of the calcineurin/NFAT dependent proteins RCAN1.4 and Cox-2 in endothelial cells.

Cox-1 has been reported to be a pro-angiogenic protein involved in processes such as tube formation in HUVEC cells (Tsujii *et al*, 1998). However, Cox-1 knockout mice showed no significant effect on the rate of tumour growth compared to the control therefore suggesting Cox-1 is not the predominant Cox isoform in angiogenesis (Williams *et al*, 2000). In contrast to Cox-2, its expression is constitutive and independent of calcineurin and VEGF-signalling in HUVEC (Hernández *et al*, 2001) and its expression was therefore studied as a control. We established over-expression of PMCA4 had no effect on the protein expression of Cox-1 compared to the control in response to VEGF-stimulation in HUVEC supporting the findings of Hernández *et al*, 2001, that Cox-2 but not Cox-1 is induced by VEGF in endothelial cells and is regulated by calcineurin signalling. Additionally, it also shows that the inhibitory role of PMCA4 is specific for processes regulated by the calcineurin/NFAT pathway. It

should be taken into consideration that a paper by Bryant *et al*, 1998, showed in HUVEC that Cox-1 levels, although constitutively expressed, were increased in response to VEGF-stimulation whereas Cox-2 expression was unaffected (Bryant *et al*, 1998). The variation in these results compared to Hernández *et al*, 2001 could possibly be due to the differences in VEGF concentration used as Bryant used a low concentration (10ng/ml) (Bryant *et al*, 1998) whereas Hernández used a higher concentration (50 ng/ml) (Hernández *et al*, 2001).

4.7.2 VEGF-Induced Cell Migration is Attenuated by Ectopic Expression of PMCA4

Endothelial cell migration is one of the processes required for successful angiogenesis (Carmeliet, 2000). VEGF-stimulation of HUVEC induces cell migration which is down-regulated by the calcineurin inhibitor CsA, suggesting this process is controlled by the calcineurin/NFAT pathway (Hernández *et al*, 2001). Additionally, RCAN1.4 and Cox-2 have been reported to be involved in the regulation of cell migration (Iizuka *et al*, 2004; Holmes *et al*, 2010; Hernández *et al*, 2001). As we previously established PMCA4 to be a negative-regulator of the calcineurin/NFAT pathway and consequently RCAN1.4 and Cox-2 expression, we wanted to investigate if this resulted in a concomitant reduction in cell migration.

Ectopic expression of PMCA4 in HUVEC resulted in a significant down-regulation of VEGF-induced cell migration. In comparison, loss of PMCA4 ($PMCA4^{-/-}$) resulted in an increase in cell migration compared to the wild-type ($PMCA4^{+/+}$), verifying that PMCA4 is a negative-regulator of VEGF-stimulated endothelial cell migration. The inhibition of migration observed in this study

could potentially be a result of the PMCA4-mediated down-regulation of RCAN1.4 and Cox-2 expression previously reported in this thesis. In support of this hypothesis two studies detected reduced endothelial cell migration in response to VEGF-signalling with knockdown of RCAN1.4 (Iizuka *et al*, 2004, Holmes *et al*, 2010). Additionally, PMA-induced migration of HDMEC and human renal microvascular endothelial cells was inhibited in the presence of a variety of Cox-2 inhibitors (Woods *et al*, 2003; Daniel *et al*, 1999). Furthermore, inhibition of VEGF-induced HDMEC migration by CsA is reversed with addition of PGE_2 (Hernández *et al*, 2001), a product of Cox-2 activity (Pai *et al*, 2001).

It should also be taken into consideration that PGE_2 produced by Cox-2 is involved in the activation of angiogenesis. It was reported in rat gastric microvascular endothelial cells (RGMEC) that PGE_2 stimulation results in an increase in the expression of VEGF at both the mRNA and protein level (Pai *et al*, 2001). VEGF has been shown to be a pro-angiogenic factor (Liekens *et al*, 2001). Therefore in light of this data, the inhibition of cell migration observed with ectopic expression of PMCA4 in this study could potentially also be attributed to the reduction in prostaglandin formation as a result of PMCA4s inhibition of Cox-2 expression at the protein and mRNA level that would consequently decrease the levels of endocrine VEGF, preventing propagation of the angiogenic response.

All the studies mentioned above demonstrate that down-regulation of the expression or activity of the pro-angiogenic proteins RCAN1.4 and Cox-2 consequently results in reduced endothelial cell migration. Our data is in support of this as we too saw inhibition of VEGF-induced HUVEC migration and

have demonstrated PMCA4 to be a negative-regulator of the expression of these two proteins.

4.7.3 VEGF-Induced *In Vitro* and *In Vivo* Tube Formation is Inhibited by Over-Expression of PMCA4

Tube formation is an additional event required for successful angiogenesis (Carmeliet, 2000). It has been reported that VEGF-induced *in vitro* tube formation of HUVEC plated onto matrigel is inhibited by CsA, suggesting a calcineurin/NFAT pathway dependent mechanism (Hernández *et al*, 2001) and has also been shown to be regulated by RCAN1.4 (Iizuka *et al*, 2004) and Cox-2 (Hernández *et al*, 2001; Woods *et al*, 2003). In light of this data and our previous findings in this study, of PMCA4s role in the inhibition of the calcineurin/NFAT pathway and consequently RCAN1.4 and Cox-2 expression, we hypothesised that tube formation could also potentially be regulated by PMCA4.

We began by studying *in vitro* tube formation of HUVEC which was found to be significantly attenuated with ectopic expression of PMCA4 compared to the control when seeded onto matrigel. Interestingly, this occurred both at the basal level and in response to VEGF-stimulation, establishing PMCA4 as a negative-regulator of tube formation. This was confirmed by knockdown of PMCA4 with si-RNA which conversely initiated an increase in tube formation that was again observed both in the absence and presence of VEGF. Similar to the hypothesis made in the case of migration, it is possible this down-regulation of tube formation with over-expression of PMCA4 is a result of inhibition of RCAN1.4 and Cox-2 expression by this membrane pump, previously reported in this

project. In support of this theory knockdown of RCAN1.4 in HDMEC resulted in inhibition of VEGF-induced tube formation in a collagen based assay, as measured by total cellular junctions and organisation (Holmes *et al*, 2010) while inhibition of Cox-2 activity by the selective Cox-2 inhibitor, rofecoxib, attenuated PMA-induced tube formation in a matrigel assay (Woods *et al*, 2003). Furthermore, addition of a Cox-2 inhibitor, NS-398, resulted in equivalent down-regulation of VEGF-induced tube formation of HUVEC to that seen in the presence of CsA which had been shown to be reversed with the addition of PGE_2, implying the involvement of Cox-2 in successful tube formation (Hernández *et al*, 2001).

However it should be noted, in contrast to our results where we observe a decrease in tube formation with ectopic expression of PMCA4, which we have reported also inhibits RCAN1.4 expression, a study by Minami *et al*, 2004 found over-expression of RCAN1 in HUVEC down-regulates VEGF- induced tube formation in a collagen gel assay (Minami *et al*, 2004). The discrepancy in results between the studies may in part be due to the experimental procedures carried out. For example, we studied tube formation after 24 hours stimulation with VEGF in comparison to 48 hours in their study. In support of this all studies mentioned above that our data is in accordance with observed tube formation between 12 and 20 hours which is similar to the time we ended the experiment in our study.

When aiming to establish the significance of a protein or molecule in a physiological or pathological setting, it is of relevance to complete *in vivo* studies where possible. In collaboration with the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones

Cardiovasculares CNIC, Madrid, Spain, Professor Redondo's group kindly investigated *in vivo* functional vessel formation and allowed the results to be included in this thesis. VEGF-induced *in vivo* tube formation was attenuated in matrigel containing Ad-PMCA4 compared to the control Ad-LacZ, as measured by a significant decrease in both haemoglobin content and the number of vessels containing blood. The data obtained in this *in vivo* study is in agreement with that observed for the equivalent *in vitro* experiments, further confirming PMCA4 as a negative-regulator of tube formation and is of particular relevance for the potential of PMCA4 as a future therapeutic target.

Interestingly, studies by Iizuka *et al*, 2004 and Hernández *et al*, 2001 have established a role for RCAN1.4 and Cox-2 in *in vivo* angiogenesis. Matrigel containing VEGF and oligonucleotides for the knockdown of RCAN1was shown to attenuate the number of vessels observed (Iizuka *et al*, 2004) while implants containing VEGF inserted into the corneas of mice resulted in reduced angiogenesis when treated with CsA, with equivalent inhibition observed in the presence of the Cox-2 inhibitor, NS-398. Additionally, negative-regulation of angiogenesis seen in the presence of CsA was reversed by treatment with PGE_2 (Hernández *et al*, 2001). This data could be used in part to explain the inhibition we observe in *in vivo* vessel formation with ectopic expression of PMCA4, as we have demonstrated this protein to be a down-regulator of the expression of the pro-angiogenic proteins RCAN1.4 and Cox-2.

However, in contrast to our own results, Qin *et al*, 2006 observed a decrease in *in vivo* angiogenesis when RCAN1.4 was over-expressed, but no effect when RCAN1.4 was knocked down in a matrigel assay (Qin *et al*, 2006). Minami *et al*, 2004 similarly found over-expression of RCAN1 reduced the vascularisation

and haemoglobin content of matrigel plugs containing VEGF (Minami *et al*, 2004).

The discrepancy in the data between these studies and our own could be due to a variation in methods used. For example, Qin *et al*, 2006 did not add VEGF but instead used a VEGF expressing tumour cell while Minami *et al*, 2004 used a lower concentration of VEGF (50 ng/plug of matrigel) compared to that used in our study (100 ng/plug of matrigel). Furthermore, the time course of experiments also varied, with Qin *et al*, 2006 observing *in vivo* tube formation after only 3 days while Minami *et al*, obtained data on day 14 after implantation, compared to the 7 days performed in this thesis. Studies that our results correlate with, previously mentioned above, such as Iizuka *et al*, 2004 and Hernández *et al*, 2001 observed angiogenesis at similar time points to our investigation such as 5 and 6 days respectively.

4.7.4 VEGF-Induced Proliferation is Unaffected by PMCA4 Expression

Successful angiogenesis also requires the proliferation of endothelial cells (Liekens *et al*, 2001). VEGF has been established to induce proliferation of HUVEC (Bryant *et al*, 1998), which is reported to occur via a calcineurin/NFAT independent pathway, as CsA has no effect on proliferation of HUVEC in response to VEGF-signalling (Hernández *et al*, 2001). Therefore we hypothesised from our previous data that identified PMCA4 as an inhibitor of

calcineurin activity, that PMCA4 will not regulate this angiogenic process as it is not calcineurin-dependent. In support of this we ascertained the proliferation of HUVEC after 3 or 6 days of stimulation with VEGF was unaffected with ectopic expression of PMCA4 compared to the control.

The MAP kinase Erk1/2 has been established as an inducer of HUVEC proliferation in response to VEGF-signalling. Furthermore, Erk1/2 phosphorylation and activity is increased in HUVEC in response to VEGF-stimulation (Yu and Sato, 1999). Therefore to confirm the above result we additionally studied the phosphorylation (activation) status of the Erk1/2 protein. Over-expression or knockdown of PMCA4 had no effect on the phosphorylation and therefore activation of Erk1/2 in response to VEGF-signalling compared to the control, concluding that PMCA4 does not regulate VEGF-induced Erk1/2 signalling in endothelial cells. This supports our original hypothesis and is in accordance with the findings of Hernández *et al*, 2001, that showed a calcineurin inhibitor did not affect proliferation of endothelial cells. As we have identified PMCA4 as an endogenous inhibitor of calcineurin and also observed no effect of PMCA4 on cell proliferation it suggests this process does not occur via signalling through the calcineurin/NFAT pathway. This data also highlights the specificity of PMCA4s inhibitory effect by seemingly only down-regulating processes regulated by calcineurin.

Interestingly, RCAN1.4 has been studied in relation to proliferation with conflicting results. For example, knockdown of RCAN1.4 in HUVEC and HDMEC has been shown to have no effect on VEGF-induced proliferation (Iizuka *et al*, 2004; Holmes *et al*, 2010) or phosphorylation of Erk1/2 in HDMEC (Holmes *et al*, 2010). Our data is in agreement with these findings as over-

expression of PMCA4 inhibits RCAN1.4 expression but has no effect on proliferation or Erk1/2 phosphorylation in response to VEGF-stimulation in HUVEC. However, a study by Qin *et al*, 2006 showed RCAN1.4 over-expression in HUVEC resulted in inhibition of proliferation with the opposite result occurring in the presence of knockdown of RCAN1.4 in response to VEGF-stimulation (Qin *et al*, 2006). Additionally, Baek *et al*, 2009 demonstrated VEGF-induced proliferation of endothelial cells isolated from RCAN1 over-expressing mice was also inhibited (Baek *et al*, 2009). The discrepancy between studies could be due to the methods adopted in each case. For example, Iizuka *et al*, 2004 and Holmes *et al*, 2010 both studied proliferation after 72 hours (one of the time points used in our experiment) whereas Qin *et al*, 2006 measured proliferation after only 24 hours and also used a lower concentration of VEGF (10 ng/ml) compared to 50 ng/ml used in other studies. Furthermore, Iizuka *et al*, 2004 and Holmes *et al*, 2010 both used methods that measure proliferation in proportion to mitochondrial activity (MTT and luminescent based cell-titre glo assay respectively). As we also used an MTT assay for measurement of proliferation this could explain the similarity between our results and their studies. In comparison Qin *et al*, 2006 measured proliferation using a thymidine incorporation assay which measures DNA synthesis in S phase and although Baek *et al*, 2009 measured proliferation after 72 hours the cells used were isolated from RCAN1 transgenic mice which could potentially identify a reason for the differences in data obtained.

4.7.5 Potential of PMCA4 as a Future Therapeutic Target

Angiogenesis is associated with a multitude of pathological phenotypes (Liekens *et al*, 2001) and is often targeted therapeutically in the treatment of a

number of these diseases (Carmeliet, 2005). As we have identified PMCA4 as an endogenous inhibitor of endothelial cell angiogenesis in this thesis, it is tempting to speculate the potential relevance of PMCA4 as a future therapeutic target in the regulation of these pathological conditions.

4.7.5a Tumour Angiogenesis

Angiogenesis is required for the propagation and survival of tumours by supplying oxygen and nutrients (Papetti and Herman, 2002) and also increases the potential of metastases (Jain, 2005). Interestingly, anti-angiogenic drugs that inhibit VEGF-signalling by either binding to VEGF such as bevacizumab or to VEGF receptors (for example Sunitinib) are being used in the treatment of colorectal cancer (Ferrara *et al*, 2004) and renal cell carcinomas (Motzer *et al*, 2006) respectively, which is hypothesised to occur due to a reduction in angiogenesis (Willet *et al*, 2004; Motzer *et al*, 2006). This data therefore highlights the advantageous blockage of VEGF-signalling in the therapeutic treatment of tumours. As we have demonstrated PMCA4 to be an endogenous inhibitor of VEGF-induced angiogenesis by reducing processes such as cell migration, tube formation and the expression of pro-angiogenic proteins our findings imply a potential role for over-expression of PMCA4 in the treatment of tumours that are VEGF-dependent, consequently inhibiting VEGF-signalling, angiogenesis and tumour growth.

It should be taken into consideration that current anti-angiogenic treatments do not directly target the tumour and are not capable of causing complete tumour regression but instead reduce and prevent further growth (Shih and Lindley, 2006). Therefore, anti-angiogenic therapies such as bevacizumab are often

administered in conjunction with chemotherapeutic agents that directly target the tumour (Hurwitz *et al*, 2004). As we have demonstrated PMCA4 to be an anti-angiogenic protein it suggests PMCA4 could be a potential therapeutic target in tumour treatment although it would need to be administered as a combined therapy for optimal treatment.

Additionally, Cox-2 has also been reported to be over-expressed in certain cancers such as cervical cancer (Kulkarni *et al*, 2001) and its expression is negatively correlated with survival and disease free survival in radiotherapy treated cervix carcinomas (Gaffney *et al*, 2001). Furthermore, xenografts of colorectal cancer cells resistant to chemotherapy treatment have an up-regulation of Cox-2 expression and tumour size compared to sensitive cells (Rahman *et al*, 2012). Lewis lung carcinomas implanted into *Cox-2*$^{-/-}$ mice have reduced tumour growth, vascular density and VEGF expression compared to wild-type, implying Cox-2 has a role in the vascularisation of tumours which is required for their growth (Williams *et al*, 2000). In support of this, Caco-2 colon cancer cells over-expressing Cox-2 had an increased rate of growth when implanted into mice compared to the control (Tsujii *et al*, 1998). Cox-2 selective inhibitors, such as Celecoxib and NS-398, have also been successfully used in the reduction of tumour growth in a variety of xenograft mouse models (Tsujii *et al*, 1998; Williams *et al*, 2000). Moreover, treatment with Celecoxib decreased tumour growth rate of chemotherapeutic resistant colorectal cancer cells in combination with chemotherapeutic agents compared to either treatment individually, which was thought to be due to an increase in apoptosis and therefore re-sensitisation of cells to treatment (Rahman *et al*, 2012).

As the above results show a beneficial role of inhibiting Cox-2 in the therapy of tumours this further implies an advantageous function of PMCA4 as a target in the treatment of tumours, as we have demonstrated PMCA4 to be a negative-regulator of Cox-2 expression.

RCAN1 has also been reported to have a role in tumour progression although its exact function varies in different cells. For example, tumour formation resulting from injection of B16 melanoma cells into mice is attenuated with adenoviral infection of RCAN1 due to a reduction in blood vessel density (Minami *et al*, 2004). Similarly, over-expression of RCAN1 in transgenic mice inhibited the growth of Lewis lung carcinoma and B15F10 melanoma cells which again was thought to be due to a reduction in microvessel density (Baek *et al*, 2009). Both these studies suggest a negative feedback role of RCAN1 on angiogenesis. The data obtained in this thesis is not in accordance with these studies as we demonstrated PMCA4 to be a down-regulator of RCAN1.4 expression and consequently angiogenic processes are inhibited. Interestingly, renal carcinoma cells injected into *RCAN1*$^{-/-}$ mice have reduced growth and microvessel density compared to the wild-type (Ryeom *et al*, 2008). Even though our results seem to support this study, in that a reduction in RCAN1.4 expression by PMCA4 consequently inhibited angiogenic processes such as tube formation, they hypothesise the negative regulation they observe is again due to a loss of negative feedback inhibition resulting in hyperactivation of calcineurin and consequently activation of apoptosis, resulting in reduced tumour growth (Ryeom *et al*, 2008), whereas we have demonstrated PMCA4 to be a negative-regulator of both RCAN1.4 expression and calcineurin activity in this project.

Therefore, further studies would need to be carried out to determine the beneficial or detrimental effect of RCAN1.4 inhibition by PMCA4 on tumour growth as the above studies suggest down-regulation of RCAN1.4 by over-expression of PMCA4 would increase tumour angiogenesis. However, we did not identify RCAN1.4 to be a negative feedback inhibitor of calcineurin and consequently calcineurin/NFAT dependent angiogenesis, therefore, there is a possibility inhibition of RCAN1.4 by PMCA4 could be used to therapeutically inhibit tumour progression.

4.7.5b Limb Ischemia

Limb ischemia occurs due to occlusion of vessels, for example due to atherosclerotic plaque formation in limbs. This can lead to adverse events such as gangrene, amputation, cardiovascular complications and in severe cases mortality. Current treatments include surgery to bypass the occlusion although this is not always successful or an option for patients. Therefore, angiogenesis has become a target in therapy of this pathology to increase perfusion of ischemic limbs (Annex, 2013).

A mouse model of limb ischemia generated by ligating the femoral artery and removing it later, demonstrated the relevance of VEGF in this pathology. VEGF expression was identified to be increased at 7 and 14 days after induction of ischemia and reduced again after this time point. Interestingly, at 7 days, blood flow through the limb was initiated and continued to recover up to 35 days after initial surgery. An increase in capillary density was also observed. Furthermore, treatment with either an angiogenic inhibitor (platelet factor-4 (PF-4)) or an antibody against VEGF resulted in equivalent inhibition of blood flow recovery,

endothelial cell proliferation, capillary density and consequently neovascularisation in the ischemic limb of mice (Couffinhal *et al*, 1998). Additionally, a study in NOD mice reported a significant attenuation of blood flow recovery and capillary density in the ischemic limb which could be reversed by treatment with an adenovirus encoding VEGF injected intramuscularly (Rivard *et al*, 1999). This data therefore demonstrates the relevance of VEGF signalling in the successful re-perfusion of ischemic limbs and its potential use in therapy for this disease (Couffinhal *et al*, 1998).

It has previously been reported in a rabbit model of limb ischemia, VEGF-A_{165} treatment via intra-arterial injection directly upstream of the occlusion results in an increase in collateral vessels compared to the control (Takeshita *et al*, 1994). Additionally, a study on one patient with limb ischemia showed treatment with plasmid DNA encoding VEGF-A_{165} resulted in an increase in collateral vessels and consequently up regulation of blood flow *in vivo* after 1 month of treatment. However, one side effect observed was oedema due to an increase in the permeability of the vessels and it should be noted the treatment was not able to reverse gangrene and amputation was required (Isner *et al*, 1996).

Furthermore, Cox-2 has been reported to have a functional role in the re-perfusion of ischemic limbs. Cox-2 expression is increased in response to induction of ischemia in wild-type mice 14 days after surgery (Ohashi *et al*, 2009). Conversely, mice containing knockout of *Cox-2* (*Cox-2*$^{-/-}$) specifically in endothelial cells had impaired recovery of blood flow and capillary density in the ischemic limb compared to the control mice (Ohashi *et al*, 2009).

As we have demonstrated PMCA4 to be an inhibitor or VEGF-signalling, Cox-2 expression and consequently angiogenesis, this data suggests up regulation of VEGF activity or Cox-2 expression could be used successfully to re-perfuse ischemic limbs. In this thesis we have reported that disruption of the interaction between PMCA4 and calcineurin or knockdown/knockout of PMCA4 increases both VEGF-induced angiogenic processes and Cox-2 expression and could therefore be used therapeutically to enhance vascularisation in ischemic limbs.

4.7.5c Endometriosis

Endometriosis is characterised by ectopic sites of endometrium that due to angiogenesis continue to grow outside of the uterus (Hull *et al*, 2003). The severity of the disease correlates with the expression levels of VEGF (Shifren *et al*, 1996) and it has been reported treatment with sFlt-1 (a soluble form of the VEGFR-1 receptor) or an antibody against VEGF, which both prevent VEGF-signalling, resulted in reduced ectopic lesion formation in a mouse model of endometriosis (Hull *et al*, 2003). Hull *et al*, 2003 proposed this down-regulation of endometriosis by inhibition of VEGF could potentially be due to an increase in apoptosis or conversely a decrease in proliferation. It should be noted, in this thesis we have not established if PMCA4 has any role in the regulation of apoptosis and therefore we cannot speculate how PMCA4 would affect this process in relation to the treatment of endometriosis. Additionally, even though we have shown PMCA4 to be a negative-regulator of VEGF-induced angiogenic processes, we established PMCA4 does not regulate endothelial cell proliferation in response to VEGF-signalling which would suggest if the improvement seen in the pathophysiology of endometriosis with inhibitors of VEGF, reported in Hull *et al*, 2003 study, was a result of inhibition of

proliferation, PMCA4 may not be the optimum therapeutic target for the treatment of endometriosis.

On the other hand, Cox-2 has also been implicated to have a role in the progression of endometriosis. Cox-2 expression was reported to be higher in eutopic endometrial stromal cells from patients suffering with endometriosis compared to the control, especially during the secretory phase of the menstrual cycle (Matsuzaki *et al*, 2004b) and has also been shown to be increased in ectopic compared to eutpoic endometrium tissue. It is proposed an increase in PGE_2 expression, due to an up-regulation of Cox-2, leads to propagation of the disease phenotype (Chishima *et al*, 2002). Additionally, treatment with a Cox-2 inhibitor, celecoxib, in a rat model of endometriosis resulted in reduced size of ectopic implants suggesting the importance of Cox-2 in propagation and maintenance of pathological endometriosis (Matsuzaki *et al*, 2004a). Furthermore, the levels of Cox-2 have also been reported to be associated with the level of dysmenorrhea (menstrual pain) with higher expression of Cox-2 correlating with more severe pain (Matsuzaki *et al*, 2004b). This suggests treatment by over-expression of PMCA4, which will consequently reduce Cox-2 expression as previously reported in this thesis, could be used to reduce growth of ectopic sites and also alleviate the pain associated with the pathology of this disease, therefore highlighting the potential of PMCA4 in the regulation of the symptoms and severity of this illness.

4.7.5d Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory disease of the joint. The synovium increases in size and develops a leading edge termed the pannus that is highly

vascularised and results in destruction of cartilage and bone. The quantity of synovial fluid also increases leading to inflammation, swelling and consequently joint pain (Paleolog, 2002). Due to the increase in proliferation required to increase mass (Maruotti *et al*, 2006) the synovia becomes hypoxic and to provide oxygen and nutrients for this additional growth angiogenesis is stimulated (Paleolog, 2002; Maruotti *et al*, 2006). It is therefore hypothesised inhibition of angiogenesis will alleviate symptoms of RA (Paleolog, 2002). It has been proposed that anti-angiogenic therapies successfully used for the treatment of tumours could potentially be utilized in RA due to similarities in angiogenic features between both diseases (Semerano *et al*, 2011).

In a mouse model of collagen induced RA, treatment with an antibody against VEGF reduced the severity of the phenotype of RA joints and postponed the onset of the disease although all mice developed arthritis (Sone *et al*, 2001a). An improvement in disease phenotype was also observed if treatment was given after symptoms of RA had already occurred (Sone *et al*, 2001a). However, in the same mouse model, one study saw an improvement in inflammation and reduced damage to bone and cartilage in the presence of an anti-VEGF antibody administered in early stages of the disease, but had no effect when given in the later stages after disease onset (Lu et *al*, 2000). The discrepancy in the efficacy of VEGF treatment after the onset of disease could be due to the time points studied as Sone *et al*, 2001a and Lu *et al*, 2000 both observed the effect of VEGF after the onset of disease but at 46 days or 50-58 days after primary immunisation respectively. In this study we have established PMCA4 to be a mediator of VEGF-induced angiogenesis by negatively-regulating processes required for angiogenesis such as cell migration and tube

formation. Therefore, over-expression of PMCA4 in this disease phenotype may relieve some of the symptoms observed, although its potential use after the onset of the disease or in its later stages is currently controversial.

Additionally, Cox-2 mRNA, protein expression and consequently PGE_2 production is elevated, whereas, Cox-1 levels remain the same in the paws of adjuvant-induced RA mouse models. Furthermore, paw oedema, number of osteoclasts and consequently bone resorption, inflammation and most interestingly Cox-2 expression is reduced in the presence of a Cox-2 selective inhibitor, SC-58125, which is a result of the reduction of PGE_2 formation. This was similar to Indomethacin, a non-selective non-steroidal anti-inflammatory drug (NSAID) that inhibits both Cox-1 and Cox-2 activity (Anderson *et al*, 1996). This data implies a role for Cox-2 but not Cox-1 in the propagation of RA. As we have demonstrated PMCA4 to be an endogenous inhibitor of Cox-2 expression, and therefore PGE_2 production (although we did not demonstrate this in this study) it suggests PMCA4 could also potentially be used successfully in the treatment of RA. It has been suggested that using a selective Cox-2 inhibitor instead of a general Cox inhibitor (that inhibits both Cox-1 and Cox-2) will reduce the adverse side effects seen with the non-selective NSAIDs (Anderson *et al*, 1996). Specifically, it has been shown in patients suffering with osteoarthritis that treatment with a selective Cox-2 inhibitor, Rofecoxib, rather than a non-selective NSAID that attenuates both Cox-1 and Cox-2 activity, resulted in reduced gastrointestinal damage often seen as a side effect when the activity of both Cox isoforms are inhibited (Langman *et al*, 1999). This further implies a beneficial use of PMCA4 in the treatment of pathological disorders as we have demonstrated PMCA4 down-regulates Cox-2 expression

but has no effect on the expression of Cox-1, therefore the reduction in severe side effects observed in the presence of Cox-2 selective inhibitors should also occur with ectopic expression of PMCA4 if used as a treatment for pathological angiogenesis.

Moreover, in an adjuvant-induced rat model of arthritis, PMCA activity was shown to be reduced and PGE_2 levels increased in lymphocytes from arthritic rats compared to the control, with the opposite result occurring after pulsed electromagnetic field (PEMF) treatment which was shown to alleviate the symptoms of RA (Selvam *et al*, 2007). This finding is highly interesting in relation to our data as we have shown an increase in PMCA4 expression decreases angiogenesis and consequently Cox-2 expression, which would reduce PGE_2 levels, and suggests the alleviation of RA symptoms which correlates with an increase in PMCA4 expression seen in Selvam *et al*, 2007 study is due to the anti-angiogenic properties of PMCA4 we have previously demonstrated in this study and further supports the potential of PMCA4 as a therapeutic target in the down-regulation of RA pathology.

However, in the study by Selvam *et al*, 2007 lymphocytes were studied whereas we used endothelial cells and PMCA activity was recorded as a whole and the involvement of individual isoforms in this case was not established. It would therefore be of relevance to distinguish the significance of the PMCA4 isoform specifically in their study as it is possible the different cells used in each study could have a different PMCA isoform involved in the result observed in each case.

It should be noted for the treatment of tumours, combined therapy such as chemotherapy and the anti-angiogenic drug bevacizumab is most beneficial to patients, targeting not only the tumour itself but also angiogenesis (Hurwitz *et al*, 2004). It is therefore hypothesised the same will apply to RA, with anti-angiogenic therapies being given in conjunction with treatments to target other significant features of RA progression such as inflammation (Semerano *et al*, 2011).

4.7.5e Psoriasis

Psoriasis is an inflammatory disease of the skin leading to hyperplasia of the epidermis (Singh *et al*, 2013). It is hypothesised angiogenesis is initiated in the psoriasis sites to cope with the high demand of oxygen and nutrients required by the increase in the number of cells (Detmar *et al*, 1994). In human psoriasis skin samples, both VEGF and the receptors VEGFR-1 and 2 have been reported to be over-expressed (Detmar *et al*, 1994) and blood and lymphatic vessels have been shown to be enlarged (Kunstfeld *et al*, 2004). Interestingly, treatment with an anti-VEGF antibody in a psoriasis mouse model resulted in inhibition of the psoriasis phenotype as observed by alleviation of swelling, inflammation and scaly skin on the paw, ear or tails, reduced quantity of inflammatory cells such as lymphocytes and macrophages, down-regulation of proliferation of keratinocytes and reduced number and size of blood vessels (Schonthaler *et al*, 2009), while treatment with antibodies against VEGFR-1 and 2 simultaneously, reduced inflammation and the size of lymphatic vessels in wild-type mice that had induced inflammation (Kunstfeld *et al*, 2004).

Interestingly, in a patient suffering with colon cancer and psoriasis, treatment with chemotherapeutic agents and the FDA approved anti-angiogenic drug bevacizumab, resulted in remission of psoriasis which was thought to be due to bevacizumab (Akman *et al*, 2008). Even though this finding is promising further investigations need to be carried out to confirm this result as only one subject was studied.

As the above data establishes inhibition of VEGF-signalling and consequently angiogenesis alleviates the symptom of psoriasis it suggests over-expression of PMCA4 could possibly be used in the treatment of this pathological disorder as we have demonstrated ectopic expression of PMCA4 to be a negative-regulator of the VEGF-induced angiogenic processes.

4.7.5f Retinopathies

There are a number of retinopathies associated with an increase in angiogenesis that leads to severe vision loss such as age related macular degeneration (AMD) (Kourlas and Abrams, 2007; Zhou and Wang, 2006) and diabetic retinopathy (Cheung *et al*, 2010). Both diseases result in vessel hyper-permeability, and in severe cases retinal detachment (Kourlas and Abrams, 2007; Cheung *et al*, 2010).

Interestingly, VEGF expression has been reported to be up-regulated in retinal endothelial cells in cases of diabetic retinopathy (Cheung *et al*, 2010) which have also been shown to have an increase in the vitreous levels of VEGF (Adamis *et al*, 1994). Therefore, anti-VEGF treatments have been used to alleviate adverse symptoms of retinopathies. For example, ranibizumab that inhibits all isoforms of VEGF (Kourlas and Abrams, 2007) has been reported in

patients with AMD to increase visual acuity and cause a decrease in choroidal neovascularisation and leakage (Brown *et al*, 2006).

Additionally, a VEGF-A_{165} selective inhibitor, pegaptanib has been successfully used to treat AMD resulting in reduced vessel permeability and neovascularisation. Advantageously, fewer adverse effects were associated with this treatment (Gragoudas *et al*, 2004).

As we have established PMCA4 as an inhibitor of VEGF-induced angiogenesis it proposes a role for PMCA4 in the therapeutic treatment of retinopathies. Over-expression of PMCA4 within the eye could potentially down-regulate angiogenesis and consequently alleviate the phenotype of both AMD and diabetic retinopathy. As we have reported PMCA4 to specifically inhibit the calcineurin/NFAT pathway in response to VEGF-stimulation it is possible fewer adverse effects will be observed if used therapeutically, as not all VEGF-signalling pathways will be down-regulated. Therefore, we hypothesise the benefits observed with the use of the isoform specific (VEGF-A_{165}) pegaptanib could also potentially occur in the presence of PMCA4.

Additionally, Cox-2 expression has been shown to be increased in the nerve fibre layer (NFL) of diabetic patients and in endothelial cells of the NFL in a mouse model of retinopathy of prematurity (ROP) in the ischemic phase. Interestingly, the use of selective Cox-2 inhibitors, APHS and etodolac attenuated preretinal neovascularisation in mouse and rat models respectively, of ischemic proliferative retinopathy, which could be reversed in the presence of PGE_2 (Sennlaub *et al*, 2003). Furthermore, Rofecoxib also reduced angiogenesis in a mouse model of ROP as determined by a reduction in the

number of blood vessels observed in the inner retina (Wilkinson-Berka *et al*, 2003). As we have demonstrated PMCA4 to be an inhibitor of Cox-2 expression in this study it suggests another potential axis of PMCA4 regulation of diabetic or AMD retinopathies. Hypothetically, over-expression of PMCA4 could be used to alleviate the phenotype of retinopathies such as that seen in the presence of Cox-2 inhibitors. However, it should be taken into consideration, Sennlaub *et al*, 2003, suggested the reduction in angiogenesis observed in the presence of a Cox-2 inhibitor was not related to VEGF-signalling (Sennlaub *et al*, 2003). As we have only demonstrated PMCA4 to be a negative regulator of VEGF-induced angiogenesis, PMCA4 may not be suitable for the inhibition of Cox-2 and consequently treatment of retinopathies. Furthermore, Cox-2 was reported to be present in both physiological and pathological retinal blood vessels and therefore inhibition of Cox-2 expression by PMCA4 may potentially be detrimental as it will not be able to distinguish between pathological and physiological vessels (Wilkinson-Berka *et al*, 2003). Therefore, the use of PMCA4 as an endogenous regulator of angiogenesis in the treatment of retinopathies in relation to Cox-2 is unclear and more studies would need to be carried out to determine the benefits.

4.7.6 Comparison of Potential PMCA4 Treatment to Current Anti-Angiogenic Therapy

Many anti-angiogenic therapies currently in use, such as bevacizumab or pegaptanib successfully utilized for the treatment of tumours (Ferrara *et al*, 2004) or AMD respectively (Zhou and Wang, 2006), function by inhibiting VEGF-signalling pathways and consequently angiogenesis (Willet *et al*, 2004; Gragoudas *et al*, 2004). As we have demonstrated PMCA4 to be an

endogenous inhibitor of VEGF-signalling via the calcineurin/NFAT pathway and consequently a down-regulator of angiogenesis, it proposes PMCA4 as a potential therapeutic target for pathological disorders requiring angiogenesis for progression, although the benefits and adverse effects of this treatment in comparison to current therapies can only be hypothesised at this time.

It has been suggested pathological angiogenesis is specifically targeted by anti-VEGF therapy as these vessels do not form correctly. One abnormality of these immature vessels compared to physiological ones is the absence of attached pericytes which makes them responsive to VEGF-signalling whereas physiological vasculature is not (Hull *et al*, 2003). As we have identified PMCA4 to be a negative-regulator of angiogenesis in response to VEGF-stimulation it suggests if PMCA4 is used therapeutically it would also be selective for pathological rather than physiological vasculature as these abnormal vessels are sensitive to alterations in VEGF-signalling.

We have also identified PMCA4 as an endogenous negative-regulator of the calcineurin/NFAT pathway. Inhibitors of this pathway such as CsA or FK506 have successfully been used in transplants to prevent rejection (Crabtree and Olson, 2002). However, its use in the treatment of other disease, such as cardiac hypertrophy in mouse models, have been less successful due to the high dosage of FK506 required that leads to severe side effects such as kidney damage (Crabtree, 1999). Therefore, PMCA4 could potentially be a superior inhibitor of calcineurin/NFAT signalling as we have identified it to be an endogenous negative-regulator of calcineurin and could possibly have less toxic effects and side effects to that seen in the presence of excess CsA or FK506.

One of the most frequent side effects observed with VEGF inhibitors is hypertension. Hypertension is thought to occur due to inhibition of NO production by eNOS as a result of down-regulation of VEGF-signalling consequently increasing blood pressure (Kamba and McDonald, 2007). As ectopic expression of PMCA4 has previously been reported to inhibit eNOS activity and consequently NO production (Holton *et al*, 2010a) it suggest hypertension could still be a serious adverse effect observed if PMCA4 is used therapeutically to inhibit angiogenesis.

A significant problem posed with current anti-angiogenic treatments is the risk of systemic adverse effects (Simó and Hernández, 2008). For example, in the case of retinopathies, such as diabetic retinopathy, anti-VEGF treatments are intravitrealy injected and therefore therapy is initially localised, however, treatment can reach the circulation and could potentially cause adverse systemic effects. Possible side effects include hypertension, proteinuria, cardiovascular complications and impaired wound healing (Simó and Hernández, 2008) with some being reported to occur as side effects of current anti-VEGF treatments, such as bevacizumab in the therapy of colorectal cancers, which then additionally also need to be treated (Hurwitz *et al*, 2004). Potentially, if PMCA4 over-expression could be localised to a specific tissue or organ involved in the particular pathology, for example by gene therapy delivered by a viral vector harbouring a tissue specific, selective promoter, angiogenesis could be inhibited with a reduction in systemic side effects possible with current anti-VEGF treatments.

VEGF has been reported to activate a variety of signalling pathways (Fig.1.1.3) such as the calcineurin/NFAT pathway (Armesilla *et al*, 1999), p38 MAPK (Yu

and Sato, 1999), Erk1/2 (Yu and Sato, 1999) and PI3K (Yu and Sato, 1999) leading to an increase in angiogenic processes such as cell migration and tube formation, reduced apoptosis and increased proliferation (Hernández *et al*, 2001; Yu and Sato, 1999). Current anti-angiogenic treatments such as bevacizumab prevent VEGF from binding to its receptor (Shih *et al*, 2006), therefore inhibiting VEGF-signalling and consequently all pathways regulated by VEGF. In comparison PMCA4 inhibits calcineurin and therefore specifically the calcineurin/NFAT pathway after stimulation with VEGF. As a result not all pathways regulated by VEGF will be inhibited, which was confirmed in this study, as we demonstrated PMCA4 had no effect on endothelial cell proliferation via the Erk1/2 signalling pathway in response to VEGF-stimulation. Interestingly, inhibition of VEGF by anti-angiogenic therapies can reduce cell proliferation and survival which is associated with some side effects such as haemorrhaging or thrombosis (Kamba and McDonald, 2007). We have reported in this thesis, VEGF-induced proliferation is unaffected by PMCA4 and therefore suggests one benefit of PMCA4 as a therapeutic target, as it would be able to inhibit pathological angiogenesis without compromising cell survival which could additionally reduce side effects observed with the use of current anti-VEGF treatments.

4.8 Conclusion

Overall we have identified a novel role of PMCA4 as a negative-regulator of VEGF-induced calcineurin/NFAT-dependent angiogenesis. Specifically, ectopic expression of PMCA4 inhibited the expression of pro-angiogenic proteins RCAN1.4 and Cox-2, cell migration and *in vitro* and *in vivo* tubular morphogenesis. Interestingly, PMCA4 expression had no effect on endothelial

cell proliferation suggesting PMCA4 could inhibit angiogenesis without compromising cell survival. Considering the number of diseases that involve angiogenesis for progression the finding in this study of PMCA4 as an endogenous negative-regulator of angiogenesis is far reaching particularly in the regulation of pathologies resulting from either insufficient or excessive angiogenesis.

5. CHAPTER FIVE

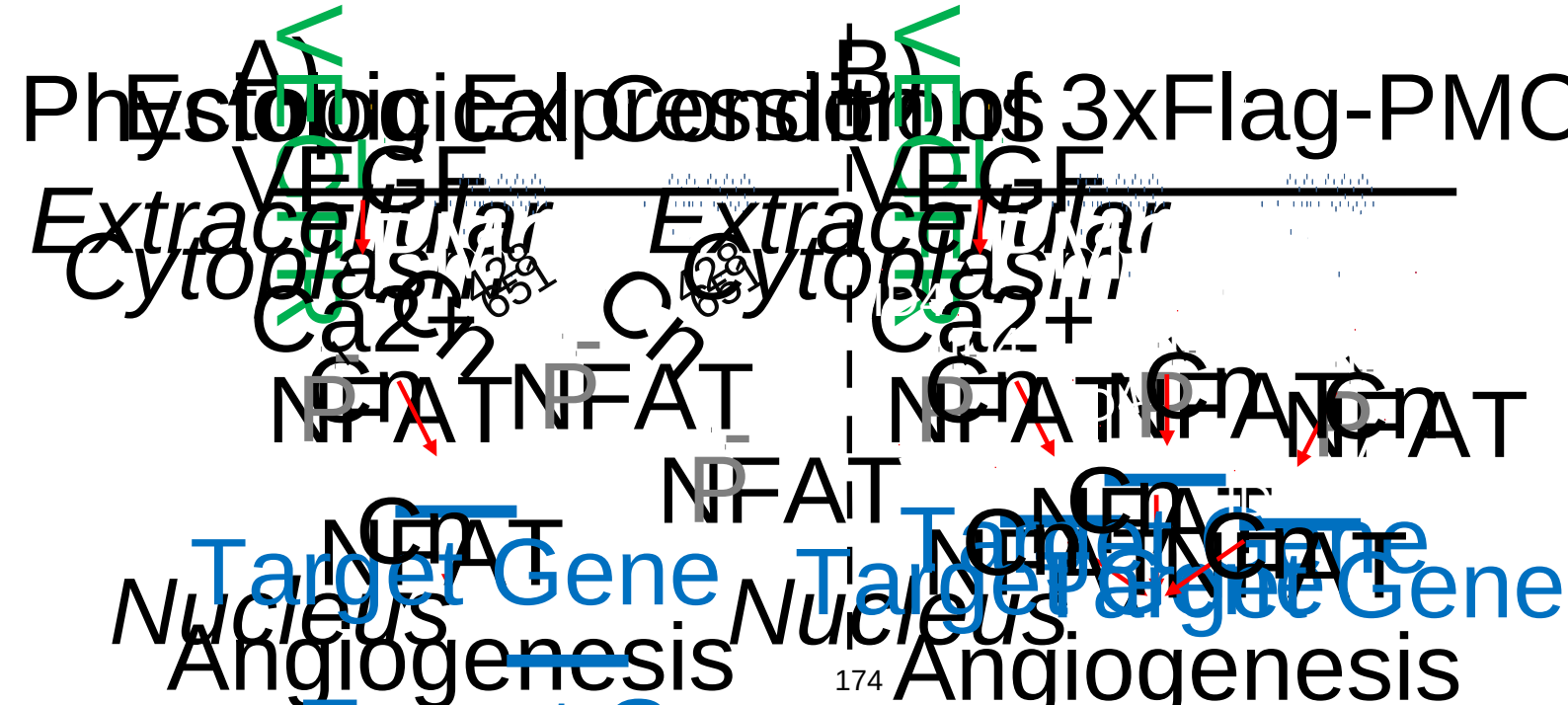
Results

Characterisation of the Molecular Mechanism of PMCA4s Inhibition of the Calcineurin/NFAT Pathway and Angiogenesis

5.1 Introduction

Previous work by our group has mapped the interaction between PMCA4 and calcineurin to the region encompassing amino acids 428 to 651 of PMCA4b (Buch *et al*, 2005). We hypothesise that PMCA4b inhibits the VEGF-mediated activation of the calcineurin/NFAT pathway via interaction with calcineurin. Over-expression of an excess of the region 428-651 of PMCA4b should compete with the endogenous PMCA4 protein in the interaction with calcineurin,

leading to disruption of the PMCA4/calcineurin interaction and therefore an increase in the activity of the calcineurin/NFAT pathway (Fig. 5.1.1).



Target Gene

Fig. 5.1.1 Hypothesis of the effect of ectopic expression of 3xFlag-PMCA4b(428-651) (Ad-ID4) on the interaction between endogenous PMCA4b and calcineurin and consequently the calcineurin/NFAT pathway. A) In physiological conditions PMCA4b and calcineurin (Cn) interact with each other. It is believed that this interaction maintains a percentage of calcineurin in a low calcium micro-environment (represented as the grey area at the membrane) which will inhibit calcineurin activity and therefore prevent NFAT translocation into the nucleus (Holton *et al*, 2010b) and consequently angiogenesis will be reduced. B) Over-expression of the region of PMCA4 that interacts with calcineurin, 3xFlag-PMCA4b(428-651) (represented as ID4 in the figure) will out-compete endogenous PMCA4b for calcineurin and therefore will prevent the interaction between the two proteins resulting in release of calcineurin from the inhibitory low-calcium microdomain, activation of calcineurin, NFAT translocation and a concomitant up-regulation of angiogenesis.

5.2 Generation of an Adenovirus Encoding the Region 428-651 of PMCA4b

As a first step to test this hypothesis we generated an adenoviral vector Ad-3xFlag-PMCA4b(428-651) (Ad-ID4) that encodes a flag-tagged version of the region 428-651 of human PMCA4b, to allow us to investigate whether the interaction between the two proteins is essential for the inhibitory effect exerted by PMCA4b on calcineurin in VEGF-stimulated cells.

The region of human PMCA4b encompassing amino acids 428-651 (numbering according to gene bank accession number: NM_001684) was amplified as described in methods using plasmid pF-PMCA4b-(428-651) as a template.

Amplification was carried out in such a way that the amplified fragment encodes three copies of the Flag epitope fused to the region 428-651 of PMCA4b.

To ensure the PCR amplification was successful and the correct fragment had been amplified PCR products were analysed by DNA electrophoresis using a 1% agarose gel. The gel showed a unique band of the expected size (797bp) suggesting that the correct region of PMCA4b had been amplified (+) (Fig. 5.2.1). A control PCR was run containing dH_2O (-) in place of the plasmid to ensure no unspecific amplification occurred in the PCR reactions (Fig. 5.2.1). Oligonucleotides forward and reverse used in the PCR reaction contained restriction sites for the nucleases BamHI and XhoI respectively to facilitate future subcloning.

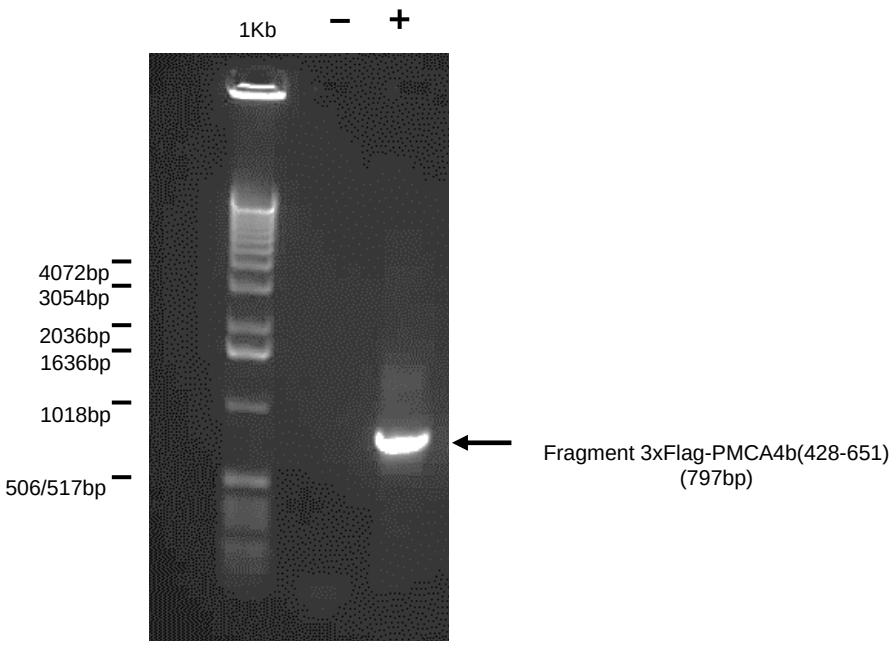


Fig. 5.2.1 Agarose gel electrophoresis of the PCR amplification of pF-PMCA4b-(428-651). DNA electrophoresis of PCR reactions performed using dH_2O (-) (negative control) or the plasmid pF-PMCA4b-(428-651) (+) (Buch *et al*, 2005) reveals a unique amplification product of the expected size (797bp) suggesting the interaction region of PMCA4 (428-651) has been amplified. A 1Kb ladder (Invitrogen, UK) was used as a marker for DNA length.

The amplified fragment was then precipitated, adenylated and ligated into the pGEM®-T Easy Vector (Promega, UK). The resulting plasmid was transformed into JM109 competent *E.coli* cells. Bacterial colonies were selected on LB-agar plates supplemented with 100 µg/ml ampicillin. Plasmid DNA was isolated by the DNA mini-preparation method from overnight cultured bacterial colonies and analysed for the presence of the fragment by restriction digestion using BamHI and XhoI enzymes. Digested samples were subsequently run in a 1% DNA agarose gel resulting in two separate bands; an upper one of 3015bp corresponding to the pGEM®-T Easy Vector and a lower one of 797bp relating

to the insert (3xFlag-PMCA4b(428-651)) (Fig. 5.2.2) All clones were positive showing successful incorporation of the insert into the vector (Fig. 5.2.2). Clone number 1 was sequenced by the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares CNIC, Madrid, Spain, to ensure the fidelity of the PCR reaction and selected for further cloning.

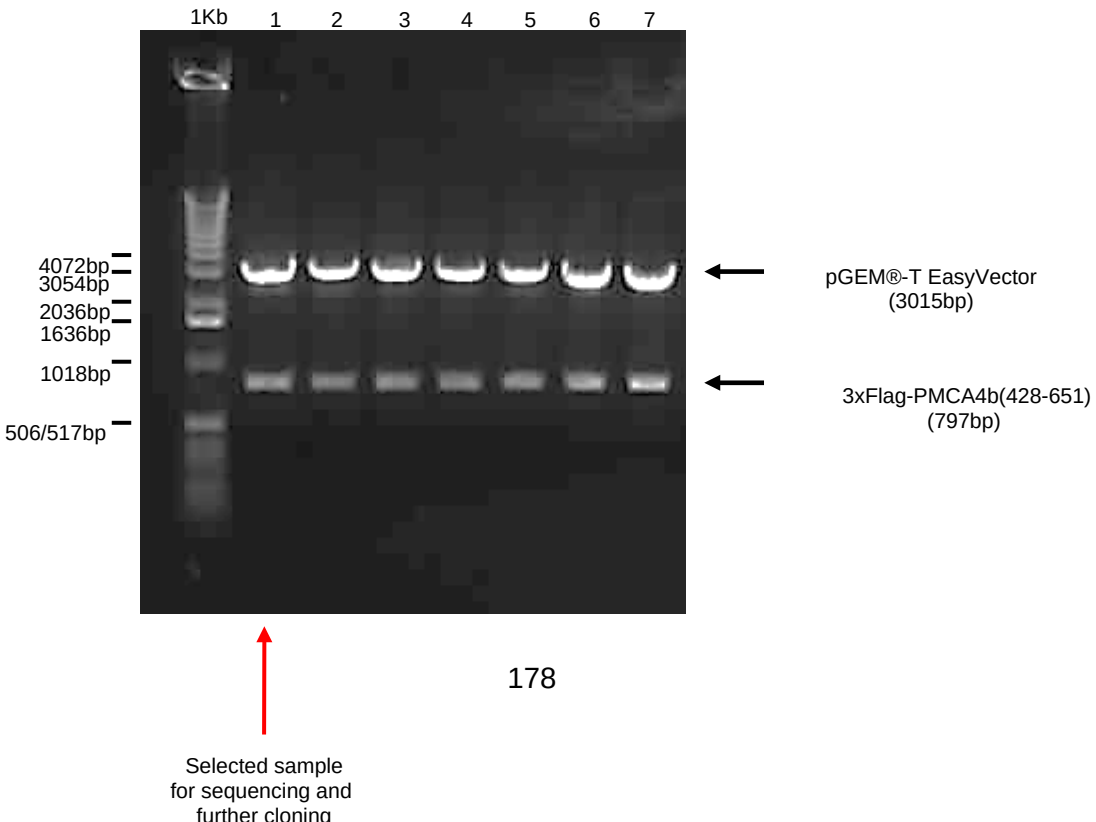


Fig. 5.2.2 Electrophoresis gel of the plasmid pGEM®-T- 3xFlag-PMCA4b(428-651) digested with BamHI and XhoI. Digestion with BamHI and XhoI of the plasmid pGEM®-T- 3xFlag-PMCA4b(428-651) with plasmid DNA obtained by DNA mini-preparation from several bacterial colonies grown overnight. Restriction digestion of the plasmid resulted in the presence of two distinct bands when run in a 1% DNA agarose gel; one of 3015bp and one of 797bp corresponding to the pGEM®-T Easy Vector and the insert (3xFlag-PMCA4b(428-651)) respectively, suggesting that the ligation procedure was successful and the vector contains the fragment. Clone 1 was sent for sequencing to confirm the fidelity of the amplified fragment and was therefore used for further cloning experiments.

The resulting plasmid was named pGEM®-T-3xFlag-PMCA4b(428-651) (Fig. 5. 2.3). Mini-preparations were then performed using clone 1 and digested with BamHI and XhoI to release the insert. The fragment BamHI- 3xFlag-PMCA4b(428-651)-XhoI (797bp) was isolated and purified after running in a 1% electrophoresis gel in preparation for its ligation with the pENTR™ 11 dual selection vector (Invitrogen, UK).

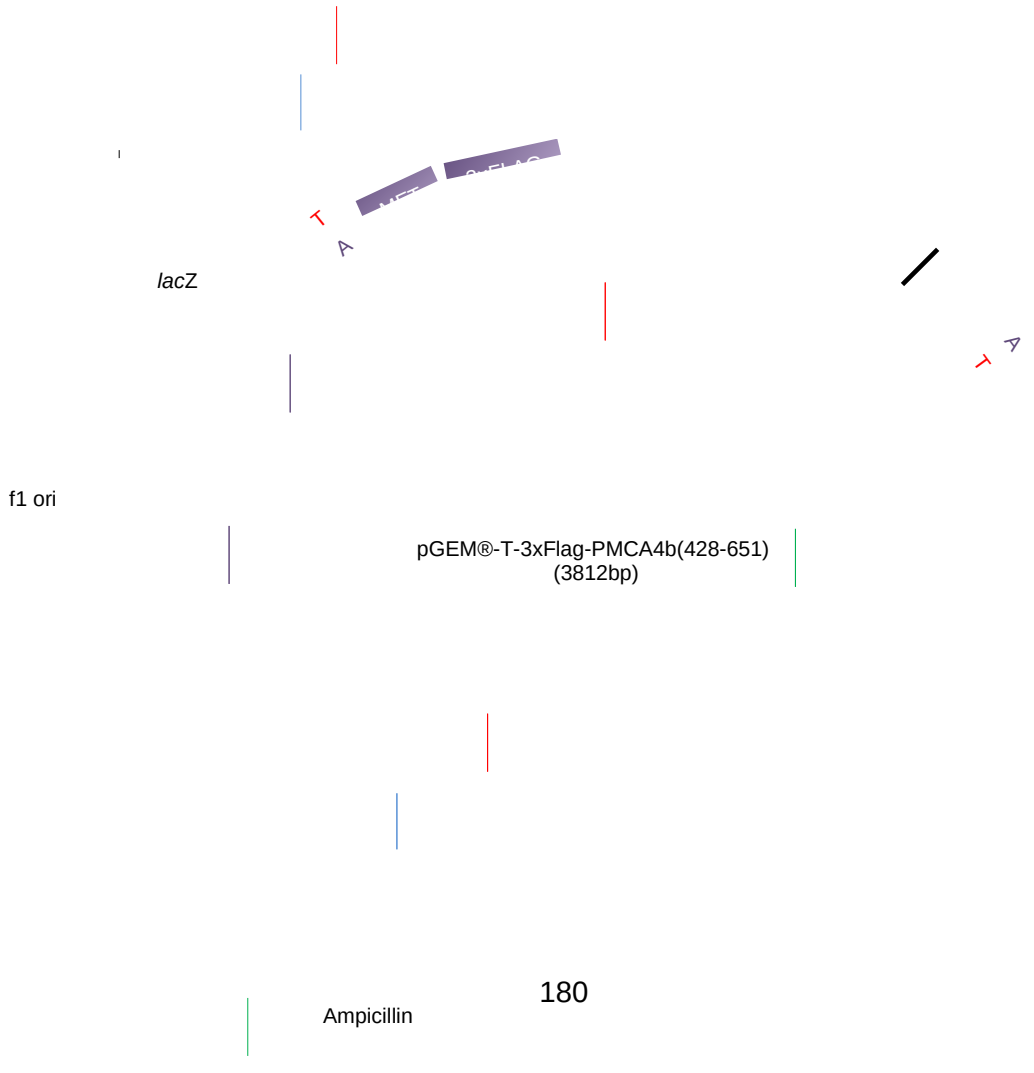

T
A
lacZ
T
A
f1 ori
pGEM®-T-3xFlag-PMCA4b(428-651)
(3812bp)
Ampicillin

Fig. 5.2.3 Schematic diagram of the pGEM®-T- 3xFlag-PMCA4b(428-651) plasmid. The plasmid pGEM®-T- 3xFlag-PMCA4b(428-651) of 3812bp generated by the ligation of the pGEM®-T Easy Vector and the insert (3xFlag-PMCA4b(428-651)). This plasmid contains the amplified fragment of PMCA4 that interacts with calcineurin (428-651) and contains a Flag tag for identification in infected cells (represented in purple in the diagram). This fragment was adenylated (A) by addition of adenosine to the 3'ends. The pGEM®-T Easy Vector contains Thymidine 3' overhangs (T) allowing efficient ligation of the vector and insert. The pGEM®-T Easy Vector contains ampicillin resistance (represented by the green band) allowing transformation and amplification of the plasmid in ampicillin (100 µg/ml) selective media and plates.

To generate an adenovirus encoding the region of PMCA4b (428-651) that interacts with calcineurin, the fragment required ligation into the pENTR™ 11 dual selection vector for incorporation into the destination vector, pAd/CMV/V5-DEST™ (Invitrogen, UK). To prepare the pENTR™ 11 dual selection vector for ligation with the fragment (3xFlag-PMCA4b(428-651)) it was digested with the enzymes BamHI and XhoI to produce complementary sticky ends to that of the insert. The vector was initially cut with XhoI and run in a 0.7% electrophoresis gel to determine successful digestion. As there is only one XhoI restriction site the vector becomes linear. Figure 5.2.4 shows a distinct band at the expected size of 3800bp when cut with XhoI. The uncut is also a single band but travels

further in the gel due to supercoiling. Therefore the digestion with XhoI was successful.

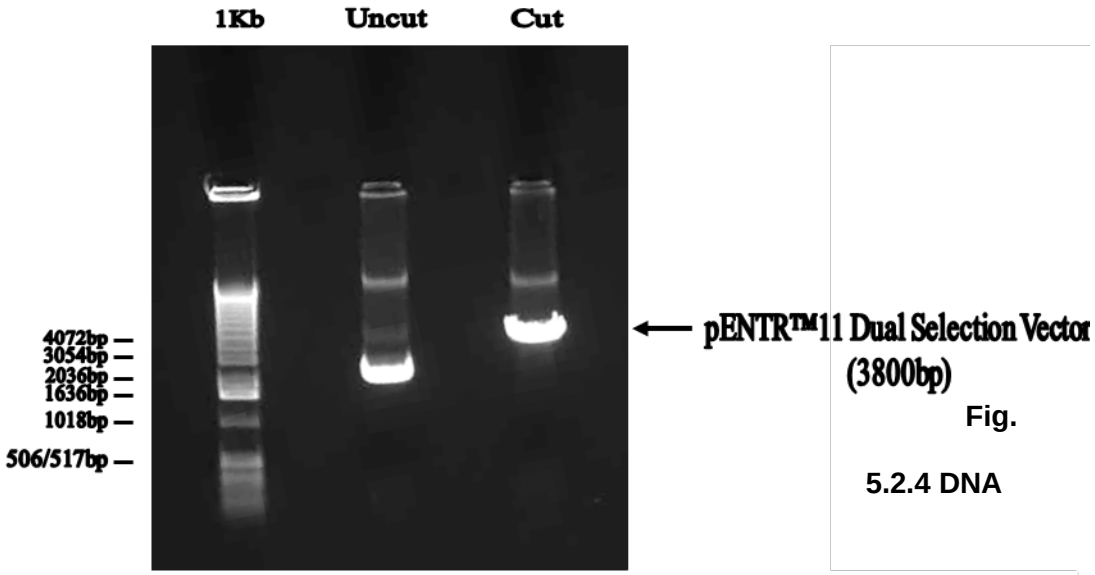


Fig. 5.2.4 DNA agarose gel of pENTR™ 11 dual selection vector cut with XhoI. The pENTR™ 11 dual selection vector was either uncut (control) or cut with XhoI and run in a 0.7%

electrophoresis gel. Digestion with XhoI resulted in a band of the predicted size of 3800bp. In comparison the uncut also generates an individual band that travels further in the gel due to supercoiling.

The digested vector was then precipitated, cut with BamHI and run in a 0.7% agarose gel. The vector contains three BamHI sites generating 3 fragments represented as an upper band of 2313bp and one lower band corresponding to fragments 693bp and 702bp which are unable to be resolved as two distinct bands due to the similarity in their size (Fig. 5.2.5). Additionally, a 92bp fragment which is between a BamHI and the XhoI restriction site would have been produced due to the previous digestion with XhoI, however, this band is not visible in the gel as the fragment is too small. The successfully digested pENTR^{TM} 11 dual selection vector was then dephosphorylated to prevent self-ligation and purified after running in a 0.7% gel. The purification was successful as only one distinct band of the expected size of 2313bp was observed (Fig. 5.2.5).

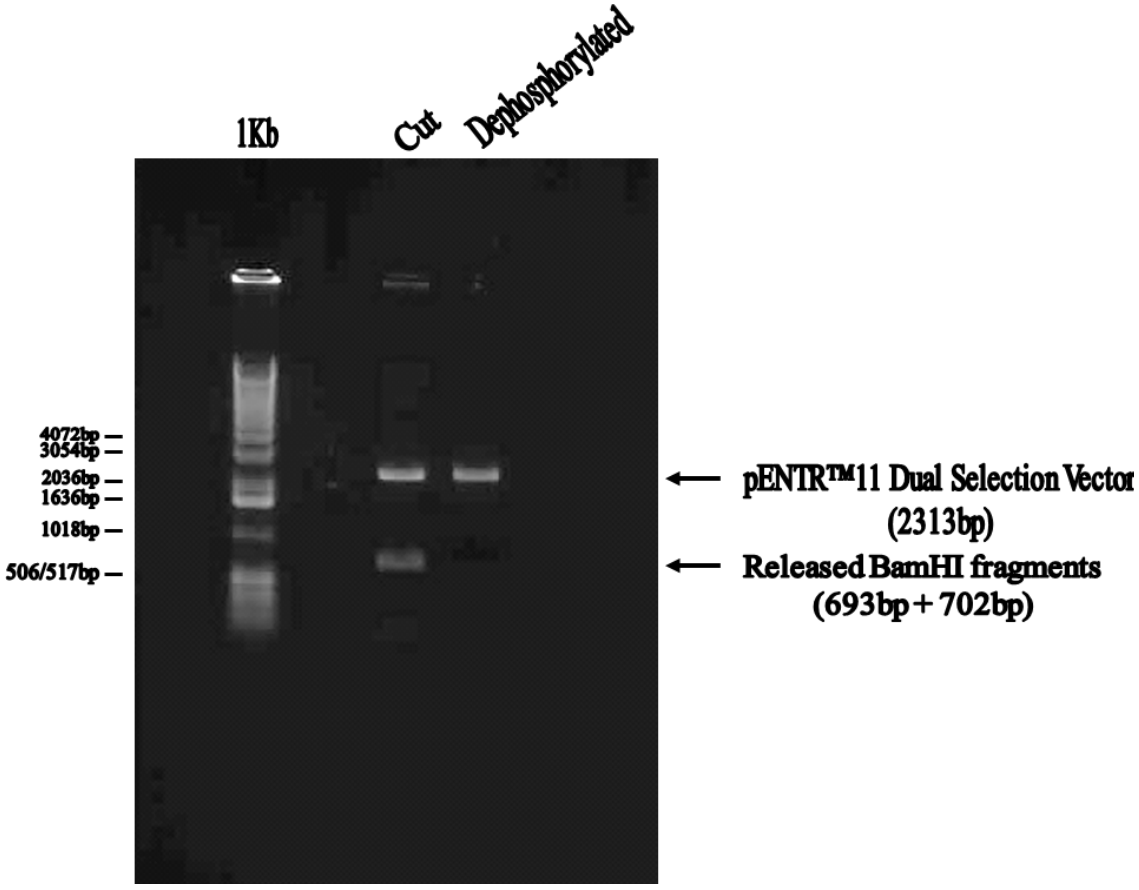

1Kb
Cut
Dephosphorylated
4072bp
3054bp
2036bp
1636bp
1018bp
506/517bp
pENTR™11 Dual Selection Vector
(2313bp)
Released BamHI fragments
(693bp + 702bp)

Fig. 5.2.5 Electrophoresis DNA gel of pENTR™ 11 dual selection vector previously cut with XhoI and digested with BamHI and dephosphorylation of this vector. Previously XhoI digested pENTR™ 11 vector was cut with BamHI generating two distinct bands at the expected position of 2313bp and a lower one corresponding to two fragments of 693bp and 702bp. The digestion was dephosphorylated and purified from a 0.7% gel which resulted in the presence of one band at the predicted position of 2313bp showing successful isolation of the digested vector that now has complementary sticky ends to the insert.

The digested and dephosphorylated vector was then ligated to the fragment 3xFlag-PMCA4b(428-651) that was also cut with BamHI and XhoI (see above), generating the plasmid pENTR™ 11-3xFlag-PMCA4b(428-651) (Fig. 5.2.6), transformed in JM109 competent cells and positive colonies selected on kanamycin (100 µg/ml) LB plates. Mini-preparations were carried out using successful clones and digested with BamHI and XhoI to check the insert was present. Cut samples were run in a 1% DNA agarose gel and produced two bands one corresponding to the vector (2313bp) and to the insert (797bp) showing that the ligation had been successful (Fig. 5.2.7)

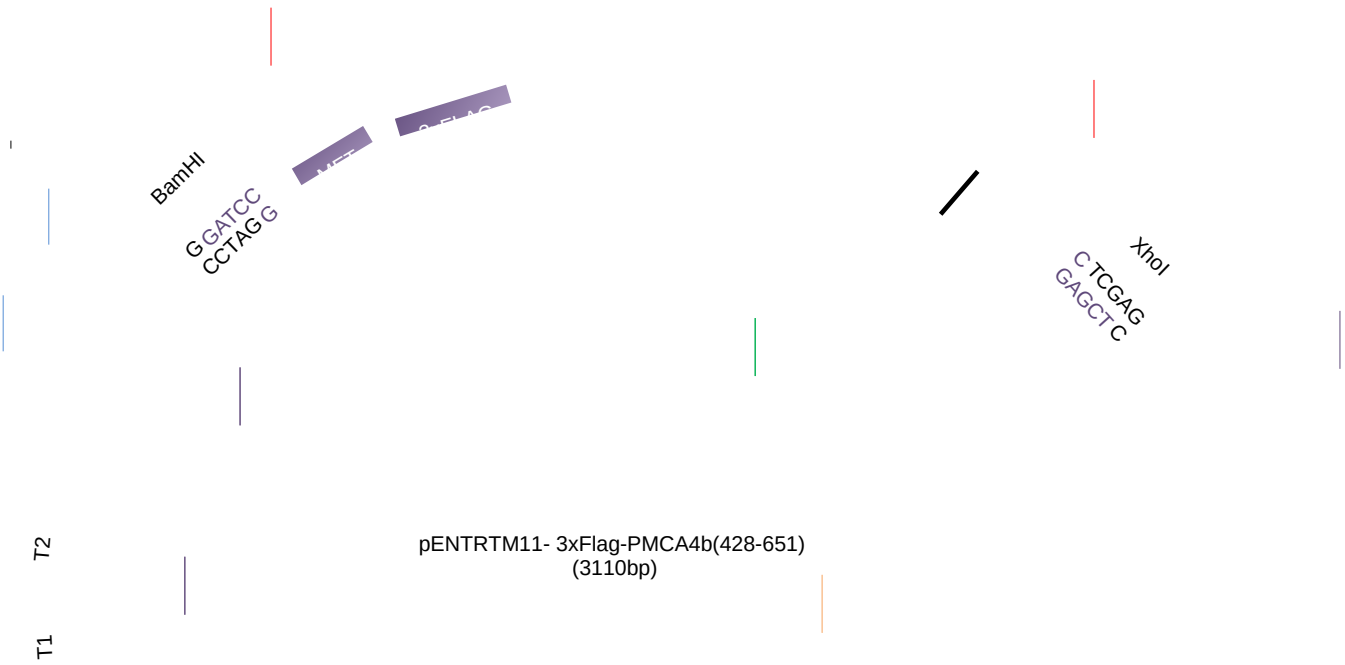


Fig. 5.2.6 Schematic diagram of the plasmid pENTR™ 11-3xFlag-PMCA4b(428-651). The plasmid of 3110bp resulting from the ligation of the pENTR™ 11 dual selection vector and the insert, 3xFlag-PMCA4b(428-651) after both have been digested with BamHI and XhoI so they

have complementary sticky ends. The plasmid contains kanamycin resistance for selective propagation of the plasmid.

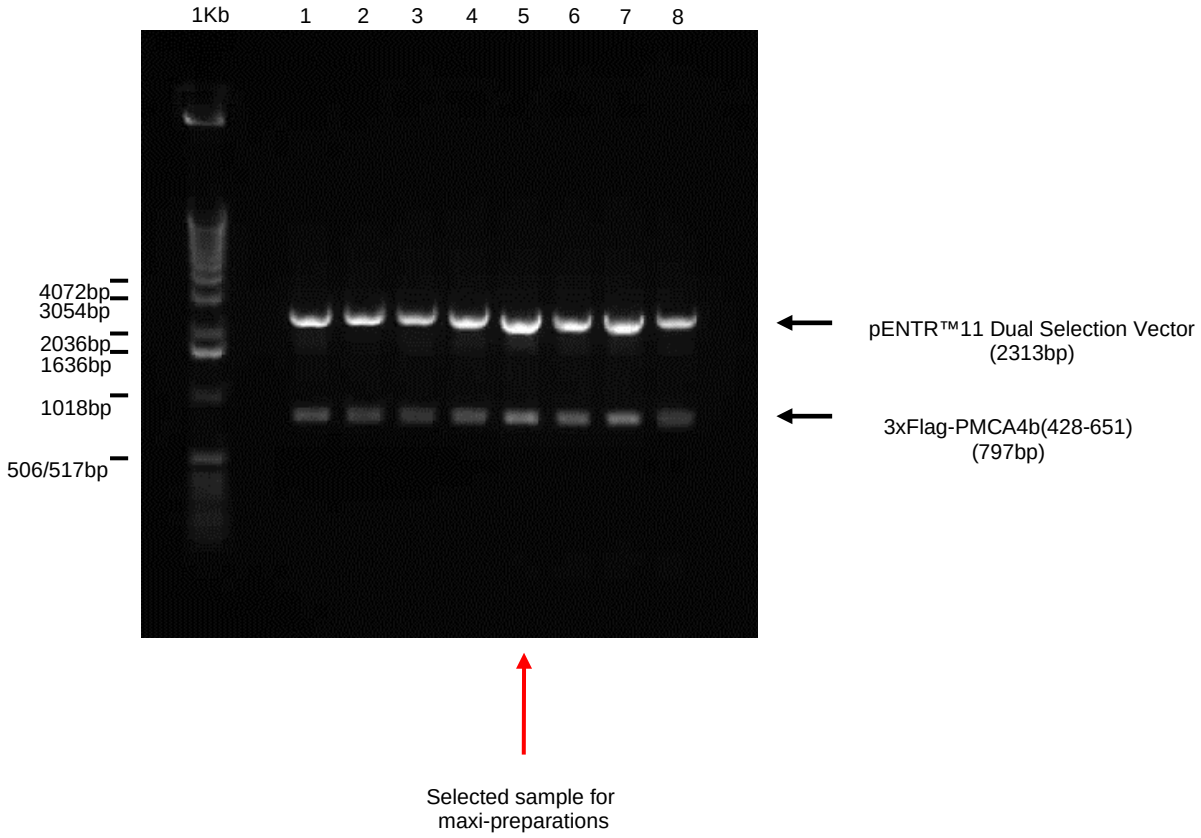


Fig. 5.2.7 Mini-preparations of the plasmid pENTR™ 11-3xFlag-PMCA4b(428-651) digested with BamHI and XhoI and run in a 1% agaose gel. The pENTR™ 11 vector and the fragment 3xFlag-PMCA4b(428-651) both digested with BamHI and XhoI were ligated and transformed in JM109 *E.coli* cells. Positive colonies were selected on kanamycin (100 µg/ml) plates and mini-preparations carried out using these colonies. Digestion of these preparations and subsequent running in a 1% electrophoresis gel

resulted in 2 separate bands; an upper one of 2313bp corresponding to the vector and a lower one of 797bp relating to the insert therefore showing ligation had been successful and the vector contained the fragment. Clone 5 was selected for further use in the cloning procedure.

Clone 5 was selected for maxi-preparations and further digestion experiments carried out with HindIII and EcoRI to confirm the vector contains the 3xFlag-PMCA4b(428-651) insert. The pENTR™ 11-3xFlag-PMCA4b(428-651) plasmid contains two HindIII restriction sites resulting in two individual bands corresponding to the plasmid (2935bp) and the HindIII fragment (175bp) after running in a 2% DNA agarose gel (Fig. 5.2.8). The uncut sample ran as one unique band showing the digestion was specific (Fig. 5.2.8).

In comparison only one EcoRI site is present in the plasmid. Running of the EcoRI digested plasmid in a 0.7% gel resulted in one isolated band at the expected size of 3110bp as the plasmid becomes linear, whereas the uncut, although still one single band, travelled further through the gel due to super coiling (Fig. 5.2.9). These digestions therefore confirm the pENTR™ 11 vector contains the insert 3xFlag-PMCA4b(428-651).

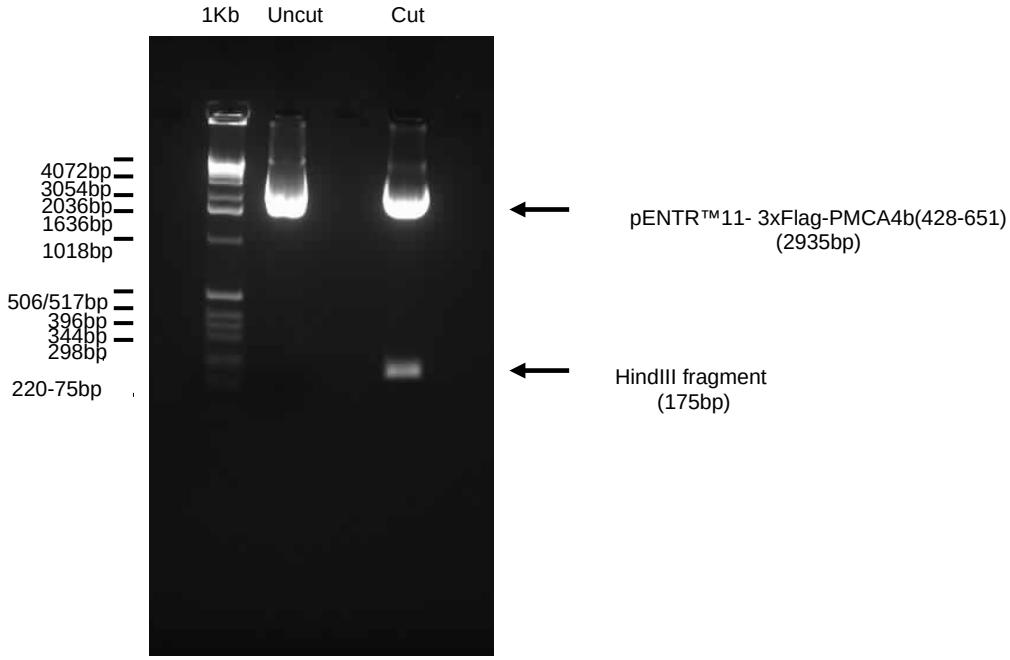


Fig. 5.2.8 A 2% DNA agarose gel of the pENTR™ 11-3xFlag-PMCA4b(428-651) plasmid cut with HindIII. Maxi-preparations of the plasmid pENTR™ 11-3xFlag-PMCA4b(428-651) were digested with HindIII and subsequently run in an electrophoresis gel (2%) resulting in two isolated bands of 2935bp and 175bp as a result of the position of the two HindIII restriction sites whereas the uncut plasmid contains one isolated band showing the digestion was specific. This therefore suggests the plasmid contains the insert 3xFlag-PMCA4b(428-651).

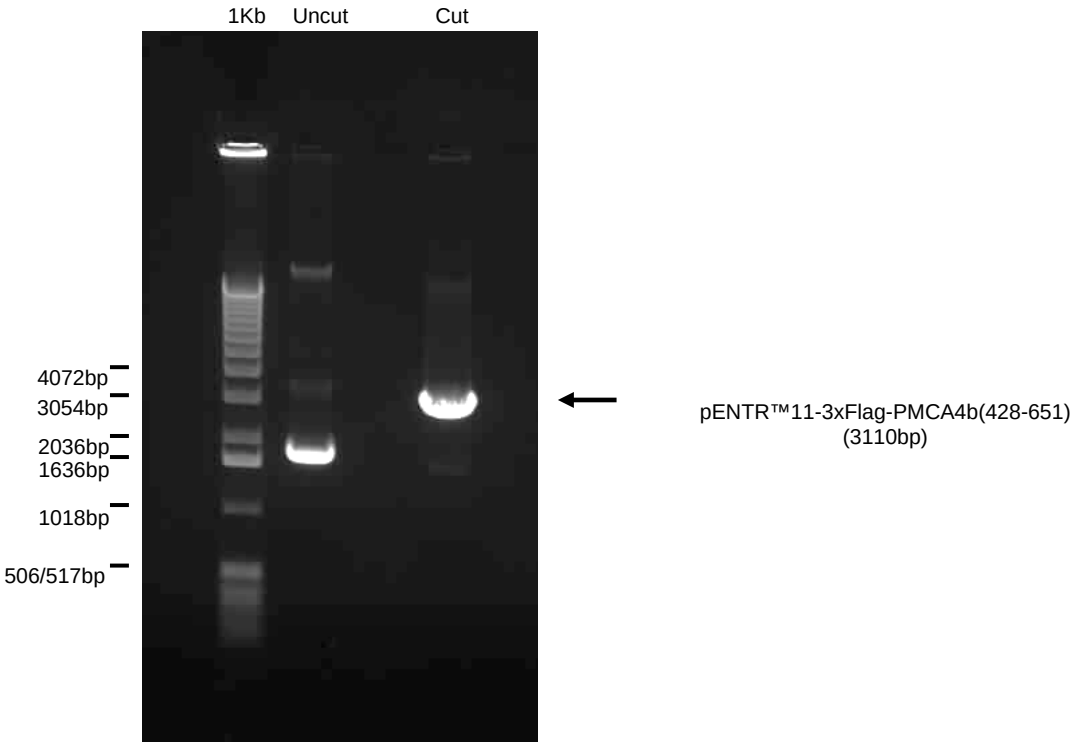


Fig.5.2.9 Digestion of the plasmid pENTR™ 11-3xFlag-PMCA4b(428-651) with EcoRI. The plasmid pENTR™ 11-3xFlag-PMCA4b(428-651) was either uncut or cut with EcoRI and run in a 0.7% electrophoresis gel. Digestion of the plasmid resulted in a unique band as expected at 3110bp due to the presence of only one EcoRI restriction site in the plasmid, therefore making it linear. The uncut plasmid travels slightly further in the gel due to supercoiling.

The pENTR™ 11-3xFlag-PMCA4b(428-651) was then recombined into the pAd/CMV/V5-DEST™ (Invitrogen, UK) using the Gateway® LR Clonase™ II enzyme mix (Invitrogen, UK), transformed and positive colonies selected on ampicillin (100 µg/ml) LB plates. The resulting plasmid was called ID4. Mini-preparations of the plasmid were digested with PacI to allow propagation of the virus in HEK293A cells. Running of the digested sample in a 0.7% electrophoresis gel resulted in two bands of the expected sizes 33257bp and 2074bp due to the two PacI restriction sites therefore confirming successful cutting with the enzyme (Fig. 5.2.10). The uncut sample had only one band showing specific digestion in the presence of the enzyme (Fig. 5.2.10). Clone 1 was sent for sequencing, confirming the presence of the insert (see appendix 5) and was therefore used for further cloning procedures.

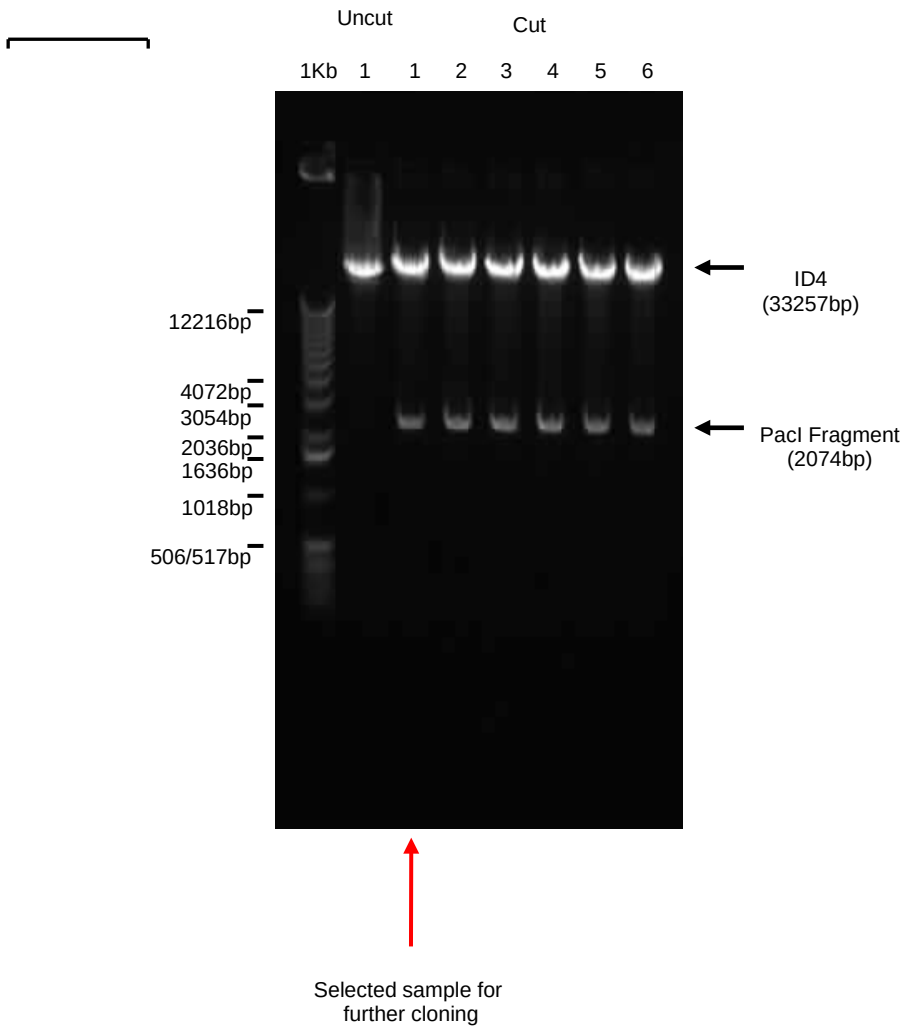


Fig. 5.2.10 Electrophoresis gel (0.7%) of the plasmid ID4 digested with PacI. Mini-preparations of the plasmid ID4 (pENTR™ 11-3xFlag-PMCA4b(428-651) recombined into the pAd/CMV/V5-DEST™ vector) digested with PacI. Cut samples resulted in two

fragments at the expected size of 33257bp (upper band) and 2074bp (lower band). The uncut (control) only had one distinct band showing the digestion was specific. Clone 1 was sequenced and confirmed to have the insert therefore this sample was used to generate the virus.

Plasmid digested with PacI was precipitated and used for the production of the primary stock of adenovirus (Ad-ID4) encoding the 3xFlag-PMCA4b(428-651) by transfection into HEK293A cells. Secondary and tertiary stocks were further obtained by infection of HEK293A with the previous stock and subsequent chloroform purification. For infection of HUVEC the tertiary stock of Ad-ID4 was used at a MOI=50 and cells were incubated for 72 hours. Due to the presence of the Flag-tag on the region of PMCA4b(428-651) that binds with calcineurin, successful infection of cells with ID4 could be demonstrated by western blot analysis using an antibody against Flag (SIGMA, UK). Infection of HUVEC with Ad-ID4 resulted in the detection of the Flag epitope by using an anti-Flag antibody. As expected no Flag recombinant protein was observed in cells infected with Ad-LacZ (Fig 5.2.11, upper panel). This result confirmed the successful generation of an adenovirus that induces the expression of the 3xFlag-PMCA4b(428-651) region that can be used to disrupt the interaction between PMCA4 and calcineurin in HUVEC. Western blots detecting PMCA4 was also carried out as a control to ensure the protein expression of the pump was not altered by transfection with the Ad-ID4 virus. Western blot using antibodies for PMCA4 (Fig. 5.2.11, middle panel) showed equivalent levels of PMCA4 protein in Ad-ID4 and Ad-LacZ infected HUVEC suggesting infection with the ID4 virus does not affect the expression of PMCA4. A western blot

detecting tubulin was also carried out to confirm equal loading of the samples (Fig. 5.2.11, lower panel).

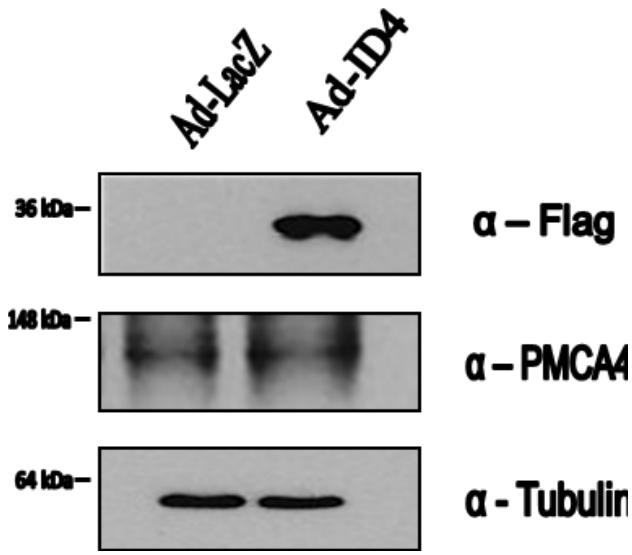


Fig. 5.2.11 Expression of 3xFlag-PMCA4b(428-651) in HUVEC. HUVEC were infected with Ad-ID4 or Ad-LacZ (control) at a MOI= 50 for 72 hours. Samples were analysed by western blot using an antibody against Flag (α-Flag). Flag was detected in endothelial cells infected with ID4 (first panel) but absent in Ad-LacZ infected cells. The expression of PMCA4 was unaffected by infection with Ad-ID4 as detected using an anti-PMCA4 antibody (α-PMCA4) compared to the control. In all cases a western blot detecting tubulin (α-Tubulin) was carried out to confirm equal amounts of protein in the samples.

5.3 Investigation of the Molecular Mechanism Involved in PMCA4-Dependent Inhibition of VEGF-Induced Calcineurin/NFAT Activity and Angiogenesis

Due to the successful generation of an adenovirus that induces the expression of the 3xFlag-PMCA4b(428-651) (Ad-ID4) corresponding to the region of PMCA4 that interacts with calcineurin we next wanted to investigate if the inhibition of the calcineurin/NFAT pathway and angiogenic processes by PMCA4 (seen in chapter 3 and 4) was a result of the interaction between the two proteins. As stated in chapter 5.1 (Fig 5.1.1) we hypothesise that expression of the interaction domain of PMCA4(428-651) in excess by infection of HUVEC with the Ad-ID4 virus will prevent the binding of calcineurin to the endogenous PMCA4 pump resulting in a lack of calcineurin inhibition. Therefore, we propose that disrupting the interaction between the two proteins will reverse the inhibitory effect observed on the calcineurin/NFAT pathway and subsequent angiogenic processes regulated by this pathway which we have seen in chapters 3 and 4.

5.3.1 The Interaction PMCA4b/Calcineurin is Essential for PMCA4 Dependent Inhibition of the Calcineurin/NFAT Pathway

We have previously demonstrated that over-expression of PMCA4 inhibits the calcineurin/NFAT pathway in endothelial cells. If this is a result of the interaction

between the two proteins, PMCA4 and calcineurin, then over-expression of the interaction region of PMCA4 (that is likely to disrupt the association between the two proteins) would prevent the inhibition observed. To test this theory HUVEC were co-infected with Ad-NFAT-Luc and either Ad-LacZ or Ad-ID4. Infected cells were then stimulated with VEGF (25 ng/ml) for 6 hours after overnight serum starvation and NFAT activity measured in a luminometer. Over-expression of the interaction region (ID4) by infection with Ad-ID4 resulted in a significant increase of 101.62%, $p \leq 0.01$, in NFAT activation compared to the control Ad-LacZ (Fig 5.3.1). This result supports our hypothesis that putative disruption of the interaction between PMCA4 and calcineurin can reverse the inhibition observed in PMCA4 over-expressing cells and therefore highlights the importance of the interaction in the mechanism of PMCA4-downregulation of the calcineurin/NFAT signalling pathway.

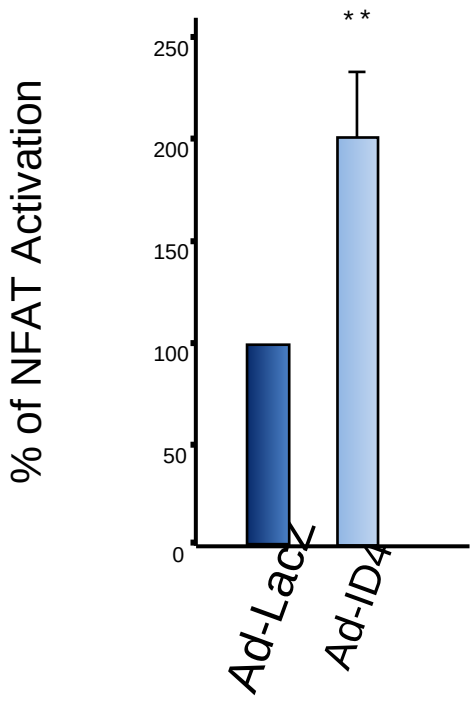


Fig. 5.3.1 Ectopic expression of the interaction domain of PMCA4 that interacts with calcineurin (428-651) increases the activation of NFAT in response to VEGF-stimulation. A) Expression of the region of PMCA4b (428-651) that interacts with calcineurin significantly increases the activity of NFAT. HUVEC were infected with Ad-LacZ or Ad-ID4. Cells were then additionally infected with Ad-NFAT-Luc, stimulated with VEGF (25 ng/ml) for 6 hours and NFAT activity measured in a luminometer. Data plotted as mean ± S.E. of three independent experiments, n=9 (** $p \leq 0.01$).

5.3.2 Ectopic Expression of the PMCA4 Interaction Domain Significantly Enhances RCAN1.4 Protein Expression in Response to VEGF-Stimulation

As we had previously identified a role for PMCA4 in the inhibition of the protein expression of the pro-angiogenic protein RCAN1.4, we wanted to determine if this inhibition could be attenuated by ectopic expression of ID4. HUVEC were infected with Ad-LacZ or Ad-ID4, serum starved overnight and analysed by western blot after 4 hours stimulation with VEGF (25 ng/ml). Western blot analysis using an antibody against RCAN1.4 (SIGMA-ALDRICH, UK) showed a significant increase of 120.24%, $p \leq 0.001$ in the expression of RCAN1.4 in cells expressing the interaction domain of PMCA4 compared to the control LacZ (Fig. 5.3.2) Equal loading of protein was confirmed by the use of an antibody against tubulin (SIGMA-ALDRICH, UK) (Fig. 5.3.2). This data also supports the theory that the interaction of PMCA4 with calcineurin results in inhibition of the calcineurin regulated expression of RCAN1.4 which can be reversed by putative disruption of the interaction between the two proteins.

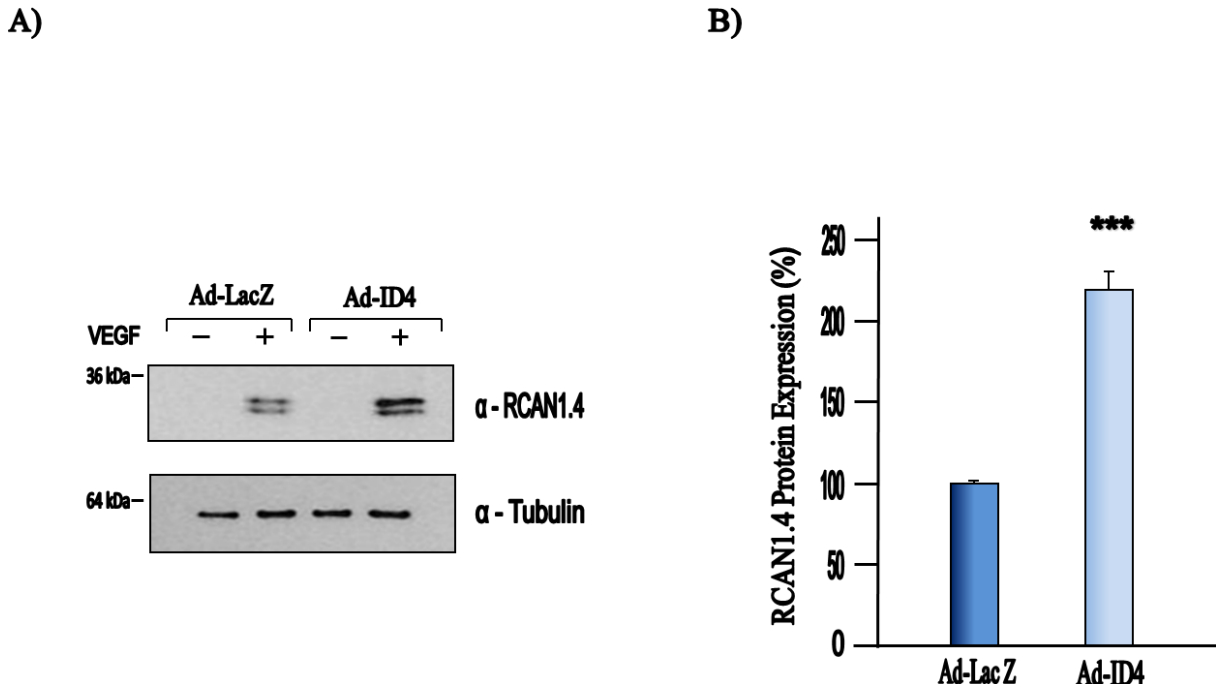


Fig. 5.3.2 Ectopic expression of the interaction region of PMCA4b, residues 428-651 (ID4), increases VEGF-induced RCAN1.4 protein expression in endothelial cells. HUVEC cells infected with Ad-LacZ or Ad-ID4 were serum starved overnight, stimulated with VEGF (25 ng/ml) for 4 hours and analysed by western blot. A) Over-expression of ID4 increases endothelial cell protein expression of RCAN1.4 (α-RCAN1.4) in response to VEGF-stimulation compared to the control LacZ. In all cases western blots detecting tubulin were carried out (α-tubulin) to show the differences observed in RCAN1.4 protein expression were not due to different amounts of protein in the samples (see appendix 14 for all western blot images used in quantification). B) Histogram shows data as mean ± S.E. of two independent experiments, n=5. Western blots were analysed using image J software (*** $p \leq 0.001$).

5.3.3 Tube Formation of Endothelial Cells is Augmented by Ectopic Expression of ID4

Tube formation both in the presence and absence of VEGF-stimulation was shown to be regulated by PMCA4. We therefore wanted to study if the inhibition in tube formation observed in the presence of PMCA4 could be reversed by preventing the interaction between endogenous PMCA4 and calcineurin by over-expression of ID4. HUVEC were infected with Ad-LacZ or Ad-ID4 and plated onto matrigel in medium 200 containing additional 2% FBS. Cells were incubated for 24 hours after which time they were fixed and images taken. The number of tubes in Ad-LacZ infected cells was set as 100% and the amount of tube formation in Ad-ID4 infected cells calculated as a percentage relative to this. Ectopic expression of ID4 significantly increased the amount of tube formation of endothelial cells by 23.23%, $p \leq 0.001$ compared to the control (Fig. 5.3.3) suggesting PMCA4 inhibition of tube formation is a result of its interaction with calcineurin.

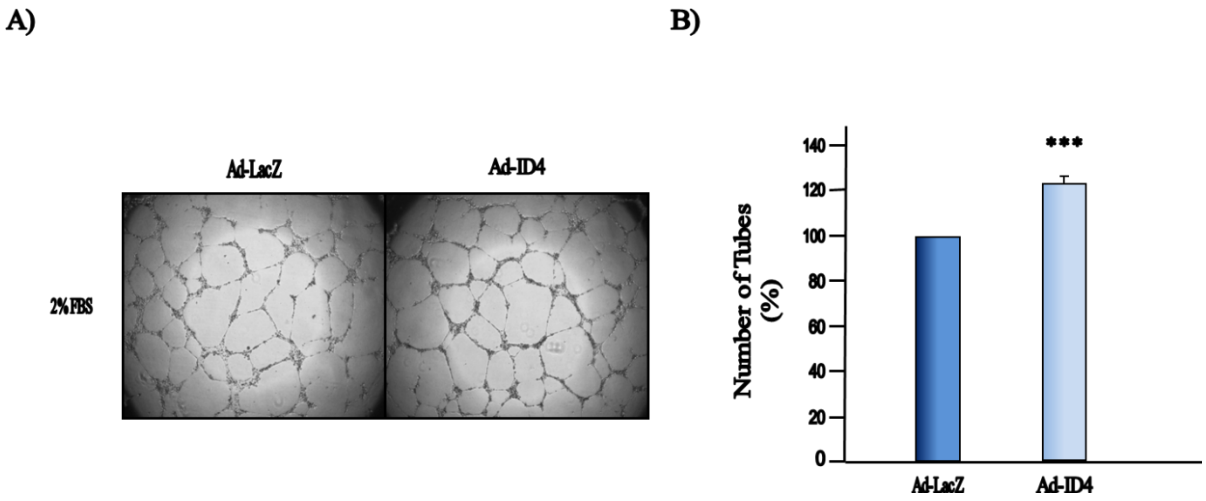


Fig. 5.3.3 Endothelial cell tube formation is increased by disruption of the interaction between PMCA4 and calcineurin. HUVEC were infected with Ad-LacZ or Ad-ID4 and plated onto matrigel in medium 200 supplemented with 2% FBS. Cells were incubated for 24 hours after which time images were taken and tube formation analysed using the Image J programme. Infection with Ad-ID4 significantly increased the number of tubes compared to the control LacZ. A) Representation of images taken and used for quantification (see appendix 15 for all images). B) Data plotted as mean ± S.E. of two independent experiments, n=8 (*** $p \leq 0.001$).

5.3.4 Over-Expression of ID4 Has No Effect on VEGF-Induced Erk1/2 Activation

Phosphorylation of Erk1/2, a MAPK protein involved in proliferation (Yu and Sato, 1999), was shown to be unaffected by over-expression or knockdown of PMCA4. We next wanted to confirm that the interaction between PMCA4 and calcineurin is not involved in the regulation of Erk1/2 activation and therefore proliferation. HUVEC were infected with Ad-LacZ or Ad-ID4 and after overnight serum starvation either left unstimulated or simulated with VEGF (25 ng/ml) for 5 minutes. Western blot analysis using an antibody against phosphorylated Erk1/2 showed no significant difference between control and Ad-ID4 infected cells suggesting the interaction between PMCA4 and calcineurin has no effect on the activation of Erk1/2 and consequently proliferation (Fig. 5.3.4). Western blots detecting total levels of Erk1/2 were carried out to normalise the phosphorylated Erk1/2 values (Fig. 5.3.4). This data confirms the finding from the over-expression and knockdown studies that PMCA4 is not a regulator of Erk1/2 phosphorylation in response to VEGF-stimulation.

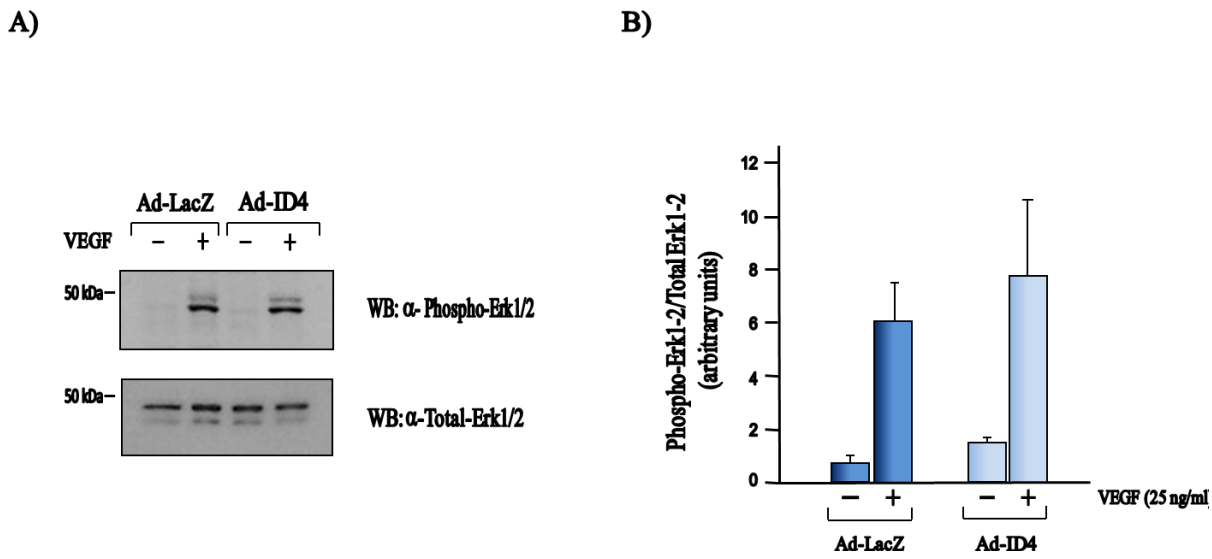


Fig. 5.3.4 VEGF-induced Erk1/2 phosphorylation is unaffected by over-expression of the interaction domain of PMCA4 (428-651) (ID4). HUVEC cells were infected with Ad-LacZ or Ad-ID4 and either unstimulated (-) or stimulated (+) with VEGF (25 ng/ml) for 5 minutes after overnight serum starvation. Western blot analysis using an antibody that detects phosphorylated Erk1/2 (WB: α-Phospho-Erk1/2) showed no significant difference between control infected (LacZ) and cells over-expressing the interaction domain of PMCA4b that interacts with calcineurin (ID4) after normalisation to the value obtained for the density of bands for western blots detecting total Erk1/2 (WB:α-Total-Erk1/2). A) Representative image of western blots used for analysis (see appendix 16 for all images) B) Histogram shows data as mean ± S.E of two independent experiments, n=3.

5.4 Discussion

It has previously been reported that PMCA4 and calcineurin interact in HEK293 cells. The interaction domain has further been mapped to the region 428-651 of PMCA4b, located in the large catalytic intracellular loop between transmembrane domain 4 and 5 (Buch *et al*, 2005). Additionally, in mammalian cells, *in vitro* over-expression of PMCA4 results in re-localisation of calcineurin from the cytoplasm to the membrane (Buch *et al*, 2005) and in neonatal rat cardiomyocytes increases the interaction with calcineurin in response to endothelin-1 (ET-1) stimulation. Furthermore, *in vivo*, an increase in association between PMCA4 and calcineurin occurs in hearts of PMCA4 over-expressing mice in response to TAC stimulation (Wu *et al*, 2009). In human breast cancer MCF-7 cells PMCA2 has also been reported to interact with calcineurin via residues 462-684 of PMCA2, the equivalent domain to 428-651 in PMCA4 (Holton *et al*, 2007).

The functionality of the association between PMCA4 and calcineurin has been demonstrated in mammalian cells. Over-expression of the interaction domain of PMCA4 (428-651) reversed the inhibition of NFAT activity seen by PMCA4 over-expression in HEK293 cells (Buch *et al*, 2005). Likewise, an increase in NFAT activity was observed in PMA/Ionophore-stimulated ZR-75-1 breast cancer cells with over-expression of the PMCA2 interaction region (462-684) (Baggott *et al*, 2012). This data implicates the interaction between the two proteins is essential for PMCAs inhibitory function of the calcineurin/NFAT pathway in mammalian cells.

It should also be noted that PMCA4 and 2 interact with other proteins such as eNOS in endothelial cells. In mammalian cells the region of association has been mapped to the same residues as those involved in the interaction with calcineurin (462-684 and 428-651 for PMCA2 and PMCA4 respectively) (Holton *et al*, 2010a). Over-expression of either PMCA4 or PMCA2 in endothelial cells resulted in inhibition of eNOS activity which could be reversed in the presence of the corresponding interaction domain for each PMCA isoform. However, it is not known if this is a result of disruption of the interaction with eNOS or calcineurin as they both bind to the same region of PMCA and calcineurin has been reported to be a regulator of eNOS activity (Holton *et al*, 2010a). In either case, it further demonstrates the role of the interaction in PMCAs regulation of partner proteins and their subsequent signalling pathways in mammalian cells.

In light of this data we decided to investigate the mechanism of PMCA4s inhibitory effect observed in this study on the calcineurin/NFAT pathway and VEGF-induced angiogenesis in endothelial cells by determining the relevance of the interaction between PMCA4 and calcineurin on these processes. To do this we generated an adenovirus that successfully expressed the interaction domain of PMCA4 (3xFlag-PMCA4b(428-651)) (ID4) in endothelial cells. Ectopic expression of the equivalent region of PMCA2b (462-684) in ZR-75-1 breast cancer cells results in reduction of membrane associated calcineurin, demonstrating the association of the two endogenous proteins is disrupted by this domain (Baggott *et al*, 2012), therefore, 3xFlag-PMCA4b(428-651) should disrupt the PMCA4/calcineurin interaction in endothelial cells. We found over-expression of ID4 and consequent putative disruption of the interaction PMCA4b/calcineurin resulted in an up-regulation of NFAT-dependent reporter

activity. This data suggests PMCA4s inhibitory function of the calcineurin/NFAT pathway is via its interaction with calcineurin. In accordance with this data ectopic expression of ID4 also increased RCAN1.4 protein expression and tube formation which are both regulated by the calcineurin/NFAT pathway (Iizuka *et al*, 2004), implying the inhibition we observe in these processes in the presence of ectopic PMCA4 is a result of its interaction with calcineurin. Interestingly, Erk1/2 phosphorylation was unaffected by over-expression of ID4 which was similar to the results observed with ectopic expression and knockdown of PMCA4, further confirming this process is not regulated by PMCA4.

Our data indicates the calcineurin inhibition observed with ectopic PMCA4 is not due to general calcium removal from the cytoplasm by the pump as the interaction domain acts as a competitor, binding to calcineurin but not interacting with wild-type PMCA4, therefore, it is unlikely to affect the calcium extrusion function of the endogenous pump and has no known calcium ejection properties itself (Baggott *et al*, 2012). If PMCA4-mediated down-regulation of calcineurin was due to general calcium clearance from the cell, inhibition of NFAT activation would still be seen in the presence of the interaction domain, however, we observe an increase in activity. It should be noted, that in the case of this study, even though we have demonstrated the interaction between PMCA4 and calcineurin to be essential for the inhibitory mechanism of PMCA4 on calcineurin activity and angiogenesis in endothelial cells we have not identified the exact function of the association. For example, PMCA4 could prevent the interaction between calcineurin and NFAT by retaining calcineurin at the membrane maintaining it out of spatial contact with NFAT (Wu *et al*, 2009) therefore attenuating NFAT dephosphorylation and further signalling via

this pathway. On the other hand, it has been hypothesised that the calcium extrusion function of PMCAs generates calcium microdomains at the membrane. Due to the association of the two proteins the calcium/calmodulin dependent calcineurin would be sequestered in a low calcium microenvironment and its activity would consequently be inhibited (Holton *et al*, 2010b). In support of the calcium micro-environment model rather than the spatial separation hypothesis, NFAT activation in response to a constitutively active calcineurin, whose activity is independent of calcium/calmodulin, was not inhibited by ectopic expression of PMCA4b (Buch *et al*, 2005). Furthermore, Wu *et al*, 2009 expressed a mutant PMCA4 pump that maintains only 15% of its calcium extrusion function in rat neonatal cardiomyocytes. This mutant only slightly reduced NFAT activity in response to phenylephrine-stimulation whereas over-expression of the wild-type pump resulted in significant inhibition (Wu *et al*, 2009). Both of these studies provide support for the calcium microdomain hypothesis as they demonstrate the importance of calcium sensitivity of the partner protein and the calcium removal function of PMCA4 for its inhibitory effect on the calcium-dependent interacting protein and consequently their signalling pathways. Even in the light of this data, our study does not definitively demonstrate that the inhibition we observe on calcineurin activity is a result of its maintenance in a low calcium microenvironment generated by PMCA4. Further work would be of interest to elucidate the exact mechanism of PMCA4 inhibition of the calcineurin/NFAT pathway and angiogenesis via interaction of the two proteins.

5.5 Conclusion

We have concluded that the interaction between PMCA4 and calcineurin is essential for its inhibitory effect on calcineurin/NFAT signalling and consequently the pro-angiogenic processes of angiogenesis regulated by this pathway, although we have not identified the exact role of the interaction. These results are potentially far reaching in the further understanding of the regulation of angiogenesis and the potential of PMCA4 as a therapeutic target in pathological angiogenesis, as disruption of the interaction can alter the regulation of angiogenic-related pathways and cellular processes.

6. CHAPTER SIX

Future Work

6.1 Further Investigation Into the Regulation of Angiogenesis by PMCA4b

In this study we have identified PMCA4 as an inhibitor of VEGF-induced calcineurin/NFAT signalling and consequently angiogenic processes regulated by this pathway in endothelial cells. The importance of this finding is far reaching due to the plethora of diseases that involve an angiogenic mechanism for progression. Future investigation into the role of PMCA4 in angiogenesis, such as studies in *in vivo* angiogenic models, would be extremely beneficial to further determine the relevance of PMCA4 and its potential as a therapeutic target for pathological angiogenesis.

6.1.1 Role of PMCA4 in *In Vivo* Angiogenesis

This project contains an *in vivo* matrigel study kindly carried out by Professor Redondo's research group at the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares CNIC, Madrid, Spain and demonstrated the inhibitory function of PMCA4 over-expression on *in vivo* VEGF-induced functional blood vessel formation. It would be of relevance to carry out a similar experiment in PMCA4 knockout mice to confirm this result. It is hypothesised injection of VEGF-containing matrigel into PMCA4$^{-/-}$ will result in an increase in functional blood vessel formation compared to the wild-type (the opposite result to that reported in this thesis for matrigel over-expressing PMCA4).

Additionally, it would be highly beneficial to study the effect of PMCA4 on pathological *in vivo* angiogenic models which are of clinical relevance and will further ascertain the therapeutic potential of PMCA4. The two *in vivo* angiogenesis models that are currently established and could be used to

substantiate the significance of PMCA4 in pathological angiogenesis are; the induced diabetic retinopathy and hind limb ischemia models which represent pathologies where the formation of blood vessels is altered by excessive and insufficient angiogenesis respectively. As we have demonstrated in this study PMCA4 is a negative-regulator of angiogenesis, we hypothesise over-expression of PMCA4 would reduce angiogenesis and therefore alleviate pathogenesis in the diabetic retinopathy model. Over-expression of PMCA4 could be achieved by local delivery of an adenovirus encoding the PMCA4 protein under the regulation of an endothelial specific promoter.

In the case of the hind limb ischemia model it would be interesting to reduce PMCA4 activity as we have shown in this project knockdown or knockout of PMCA4 enhances angiogenesis. The same result can be achieved by disruption of the interaction between endogenous PMCA4 and calcineurin by ectopic expression of the PMCA4 interaction region (428-651).

6.1.2 Recovery Experiments with PMCA4

We have demonstrated that knockout of PMCA4 in MLEC or expression of the interaction domain of PMCA4 (to reduce its association with calcineurin) in HUVEC results in an increase in RCAN1.4 protein expression in response to VEGF-stimulation. It has been reported in ZR-75-1 cells expression of the interaction region of PMCA2 reduces cell viability which can be recovered when PMCA2 is co-ectopically expressed to re-establish its interaction with calcineurin and therefore inhibit its activity (Baggott *et al*, 2012).

To further confirm that PMCA4 is an inhibitor of angiogenesis, recovery experiments similar to the one carried out by Baggott *et al*, 2012 can be used in

the above two models by adenovirus-mediated over-expression of PMCA4 to ascertain if we restore inhibition of RCAN1.4 protein expression. We predict that transfection of PMCA4$^{-/-}$ MLEC with an adenovirus encoding PMCA4 or co-expression of the interaction domain and PMCA4 in HUVEC will recover inhibition of VEGF-induced RCAN1.4 protein expression.

6.2 Investigating the Significance of Other PMCA Isoforms in the Regulation of Calcineurin/NFAT-Dependent Angiogenesis

It has previously been shown PMCA4 is expressed and interacts with calcineurin in endothelial cells (Holton *et al*, 2010a).We have demonstrated in this thesis that PMCA4 is a negative-regulator of calcineurin and the calcineurin/ NFAT pathway. This results in concomitant down-regulation of angiogenic processes regulated by this pathway. Other PMCA isoforms such as PMCA1 are expressed and also interact with calcineurin in endothelial cells (Holton *et al*, 2010a). Therefore, further work in ascertaining the significance of other isoforms in the regulation of calcineurin and consequently angiogenesis is of particular interest.

Dr Armesilla's laboratory is currently investigating the effect of PMCA1 knockdown on the regulation of the calcineurin/NFAT pathway and angiogenesis. It has been suggested that the presence of more than one isoform of PMCA can result in redundancy in the role of a single isoform as an inhibitor. For example, Holton *et al*, 2010a established knockdown of PMCA4 in endothelial cells by shRNA had no effect on basal or acetylcholine-induced NO production suggesting loss of one isoform of PMCA is compensated for by the presence of alternative isoforms (Holton *et al*, 2010a). In relation to our data,

knockdown or knockout of PMCA4 resulted in an increase in angiogenic processes, however, it is unknown if other isoforms present are partially compensating for the absence of PMCA4. Knockdown of multiple isoforms could potentially further enhance the increase in angiogenesis we observed with loss of PMCA4 expression alone.PMCA1 and 4 are the major isoforms expressed in endothelial cells, therefore the role of PMCA1 in the regulation of VEGF-induced angiogenesis needs to be established. This can be accomplished by knockdown studies similar to that carried out for PMCA4. If PMCA1 is shown to be a regulator of the calcineurin/NFAT pathway and consequently angiogenesis it would be interesting to study simultaneous knockdown of PMCA1 and PMCA4. Unfortunately PMCA1 knockout studies and therefore *in vivo* models pose a challenge as loss of PMCA1 is embryonic lethal. A conditional knockout of PMCA1 would need to be generated. It should be taken into consideration that the localisation of PMCAs has been shown to vary at the membrane. For example, it has been reported in pig cerebellum synaptic plasma membranes that only the PMCA4 isoform localises to lipid rafts (Sepúlveda *et al*, 2006). As lipid rafts have been proposed to be the site of signalling complex formation (Simons and Toomre, 2000) it suggests PMCA4 could regulate signalling complexes produced here. In support of this a recent study has reported disruption of lipid rafts in HEK293 cells prevented the interaction between PMCA4 and nNOS (Duan *et al*, 2013). Schuh *et al*, 2001 have previously shown that the interaction between the two proteins inhibits nNOS activity (Schuh *et al*, 2001). Together this data implies the localisation of PMCA4s in lipid raft fractions of the membrane is required for its regulation of signalling pathways such as nNOS. As PMCA1 has been shown to not localise

in lipid rafts (Sepúlveda et al, 2006) there is the possibility it may not be a regulator of signalling pathways. Therefore it would be very interesting to determine the exact involvement of PMCA1 in the regulation of the calcineurin/ NFAT pathway and consequently angiogenesis in endothelial cells.

6.3 Conclusion

The findings of this study are far reaching, not only demonstrating PMCA4 as a regulator of signalling pathways in endothelial cells, but also identifying a novel role for this protein in the regulation of angiogenesis. Further study into the role of PMCA4 and potentially other isoforms in the regulation of signalling pathways in endothelial cells, provides a potential endogenous therapeutic target for the regulation of pathological angiogenesis that could benefit the treatment of a multitude of diseases.

Concluding Remarks

This thesis has clearly established the following:

1- PMCA4 is a negative-regulator of the calcineurin/NFAT pathway in endothelial cells in response to VEGF-stimulation.

2- PMCA4 inhibits the calcineurin/NFAT dependent pro-angiogenic proteins RCAN1.4 and Cox-2 at both the protein and mRNA expression levels after stimulation with VEGF, but does not affect the constitutively expressed and calcineurin-independent Cox-1 protein.

3- Endothelial cell migration is inhibited by ectopic expression of PMCA4 and upregulated by suppression of PMCA4 expression.

4- *In vitro* and *in vivo* endothelial cell tube formation is inhibited by over-expression of PMCA4 in response to VEGF-signalling.

5- VEGF- induced endothelial cell proliferation is unaffected by ectopic expression of PMCA4.

6- Over-expression or knockdown of PMCA4 does not affect the phosphorylation of Erk1/2.

7- Disruption of the interaction between PMCA4 and calcineurin in endothelial cells by over-expression of the interaction region of PMCA4b (428-651) (ID4), increases calcineurin/NFAT signalling and consequently RCAN1.4 protein expression and *in vitro* tube formation, in response to VEGF-stimulation.

8- Ectopic expression of ID4 has no effect on VEGF-induced Erk1/2 phosphorylation.

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Appendix

APPENDIX 2: Table of oligonucleotides used for PCR amplification and the corresponding percentage of gel required to visualise bands (size of bands measured in base pairs (bp))

Amplification Of	Template	Forward (Sense)	Reverse (Antisense)	Annealing	Gel	Size of
Mouse PMCA1 (mRNA)	MLEC cDNA (M					370
Mouse P (mRN						
Mouse I (mRN						A
3xFlag PM (428-6						A
						T
						gT
						AT

Protein of Interest	Molecular Weight (kDa)	SDS-PAGE (%)	Primary Antibody	Host	Dilution	Secondary Antibody	Host	Dilution
RCAN1.4	22.85	12	Anti-DSCR1 (C-terminal) (SIGMA-ALDRICH)	Rabbit	1:1000	Anti-Rabbit IgG (whole antibody) (GE Healthcare)	Donkey	1:5000
Cox-1	71	8	Anti-Cox-1 (Alexis® Biochemicals)	Mouse	1:1000	Anti-mouse IgG (whole molecule) (SIGMA)	Sheep	1:5000
Cox-2	71	8	Anti-Cox-2 (Alexis® Biochemicals)	Mouse	1:1000	Anti-mouse IgG (whole molecule) (SIGMA)	Sheep	1:5000
Tubulin	55	8	Anti-α-Tubulin (SIGMA-ALDRICH)	Mouse	1:2500	Anti-mouse IgG (whole molecule) (SIGMA)	Sheep	1:5000
Erk1/2 (phosphorylated)	42,44	10	Anti-phospho specific Erk1/2 (Cell Signalling)	Rabbit	1:2500	Anti-Rabbit IgG (whole antibody) (GE Healthcare)	Donkey	1:5000
Erk1/2 (Total)	42,44	10	Anti-Erk1/2 (Cell Signalling)	Rabbit	1:2500	Anti-Rabbit IgG (whole antibody) (GE Healthcare)	Donkey	1:5000
PMCA1	135-140	6	Anti-PMCA1 (Swant)	Rabbit	1:10000	Anti-Rabbit IgG (whole antibody) (GE Healthcare)	Donkey	1:5000
PMCA4b	135-140	6	Anti-PMCA4 (Swant)	Rabbit	1:25000	Anti-Rabbit IgG (whole antibody) (GE Healthcare)	Donkey	1:5000
PMCA4b	135-140	6	JA3 (Santa Cruz)	Mouse	1:1000	Anti-mouse IgG (whole molecule) (SIGMA)	Sheep	1:5000
3xFlag –PMCA4b (428-651)	27.6	12	Anti-Flag M2 peroxidase (HRP) conjugate (SIGMA)	Mouse	1:2000	-	-	-

APPENDIX 4: Table of enzymes and the corresponding buffer used for restriction digestions, the percentage of DNA agarose gels required for visualisation of digested fragments and the size of the bands observed as measured in base pairs (bp)

pGEM®-T- 3xFlag PMCA4(428-651)	BamHI + XhoI	B	1	3015 + 797
pENTR™ 11 dual selection vector	XhoI	D	0.7	3800
pENTR™ 11 dual selection vector digested with XhoI	BamHI	E	0.7	2350 + 693 + 702
pENTR™ 11- 3xFlag PMCA4(428-651)	BamHI + XhoI	B	1	2350 + 797
pENTR™ 11- 3xFlag PMCA4(428-651)	HindIII	E	2	2350 + 175
pENTR™ 11- 3xFlag PMCA4(428-651)	EcoRI	H	0.7	3147
pAD-ID4	PacI	1	0.7	2074 + 34612

APPENDIX 5: Sequencing confirming the successful generation of pAD-ID4 for Ad-ID4 production. Performed by the Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares CNIC, Madrid, Spain

ALA A (G3-0E7CV 12524-019)

Fri Dec 28, 2012 14:14 +0100

G3-0E7CV_12524_019.ab1 from 1 to 1298

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Blast

Homo sapiens ATPase, Ca++ transporting, plasma membrane 4 (ATP2B4), transcript variant 2, mRNA

Sequence ID: ref|NM_001684.4|Length: 8753Number of Matches: 1
Related Information
Gene-associated gene details
UniGene-clustered expressed sequence tags
Map Viewer-aligned genomic context
Range 1: 2179 to 2852GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
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Fri Dec 28, 2012 15:53 +0100

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    163 GAT GAC AAG CTT GCG GCC GCG AAT TCC GCT GTC ACC ATC TCA CTG GCC TAC TCT GTG AAG

     21 Asp Asp Lys Leu Ala Ala Ala Asn Ser Ala Val Thr Ile Ser Leu Ala Tyr Ser Val Lys


    223 AAA ATG ATG AAA GAC AAT AAC CTA GTA CGG CAC TTG GAT GCT TGT GAG ACC ATG GGC AAC

     41 Lys Met Met Lys Asp Asn Asn Leu Val Arg His Leu Asp Ala Cys Glu Thr Met Gly Asn


    283 GCC ACC GCC ATC TGC TCT GAT AAG ACA GGC ACG TTG ACC ATG AAC CGC ATG ACT GTG GTA

     61 Ala Thr Ala Ile Cys Ser Asp Lys Thr Gly Thr Leu Thr Met Asn Arg Met Thr Val Val


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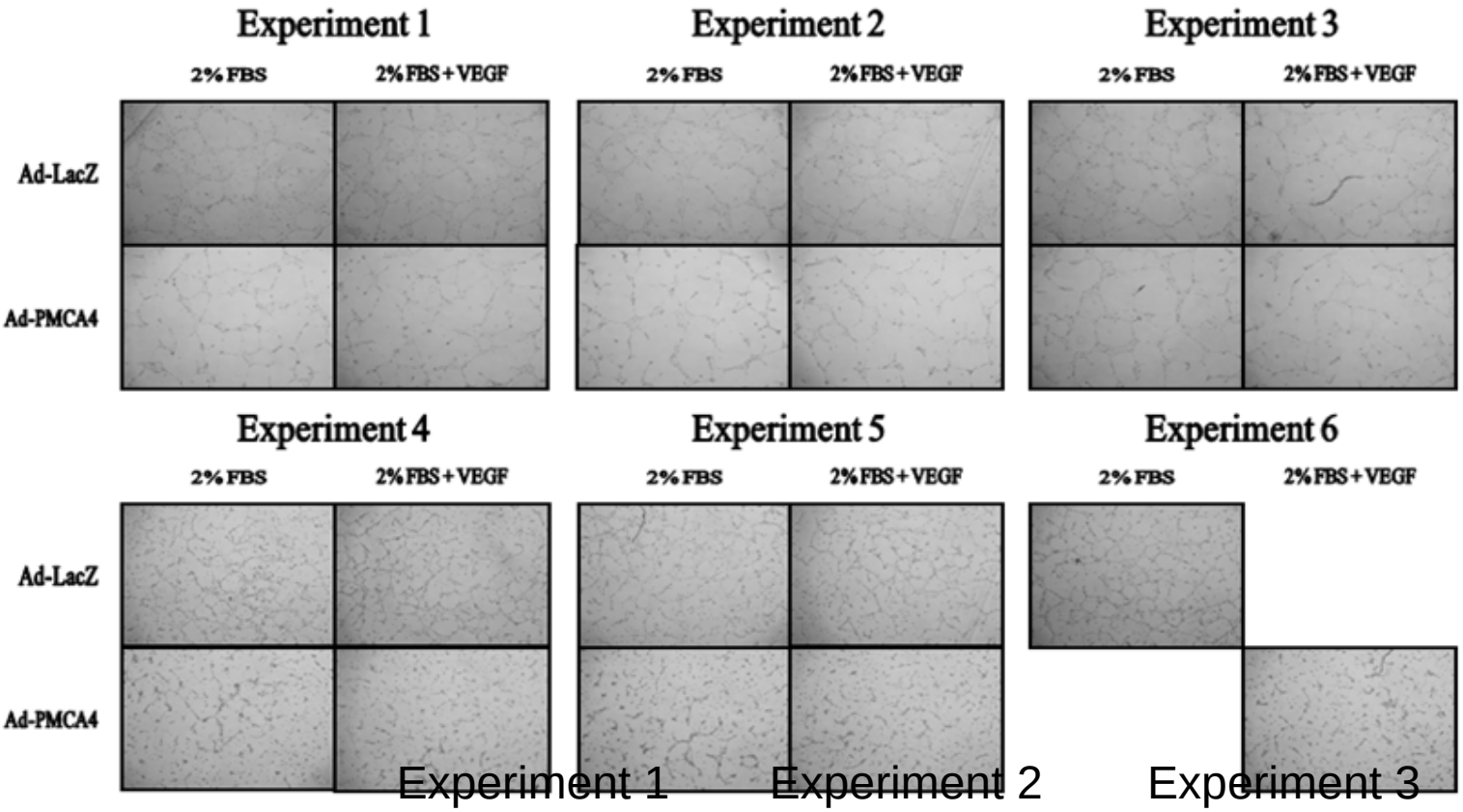
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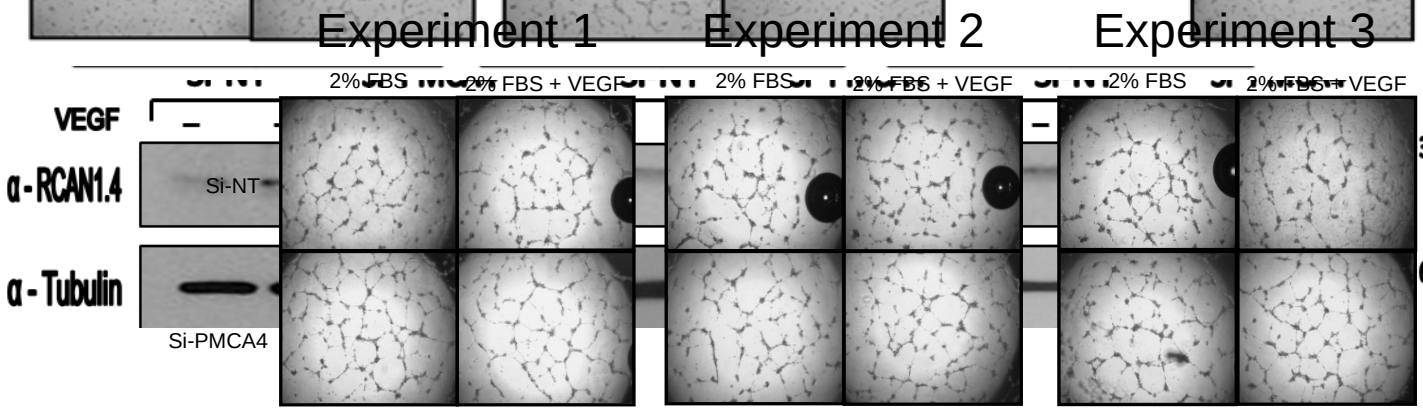
Experiment 2
Experiment 3
2% FBS
2% FBS + VEGF
Ad-LacZ
Ad-PMCA4
Experiment 4
Experiment 5
Experiment 6


Experiment 1
Experiment 2
Experiment 3
2% FBS
2% FBS + VEGF
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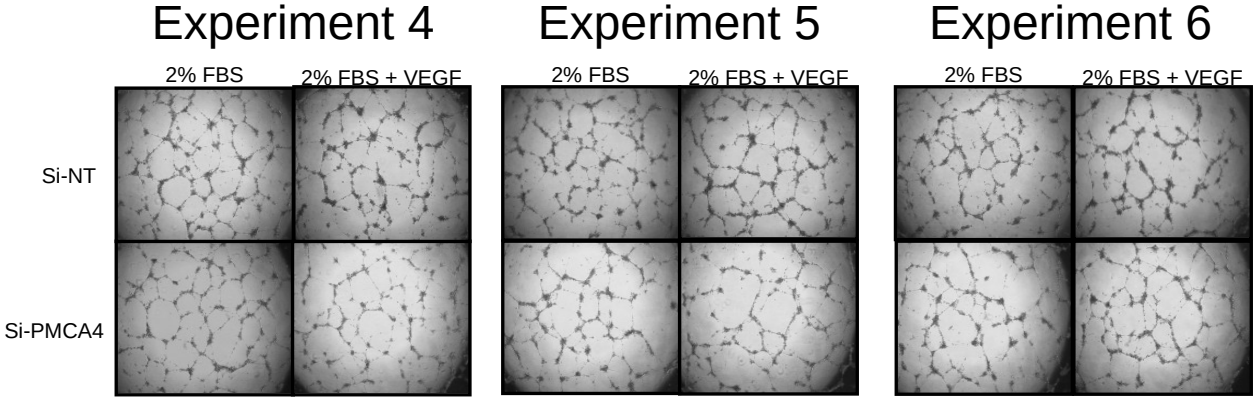
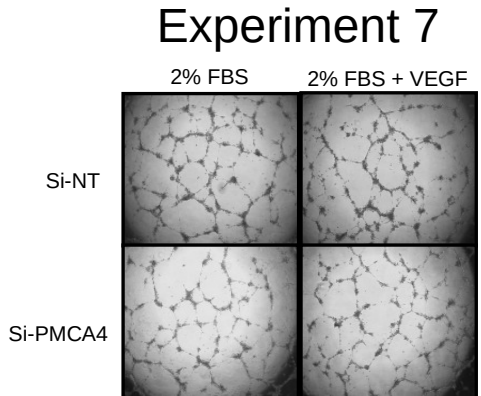
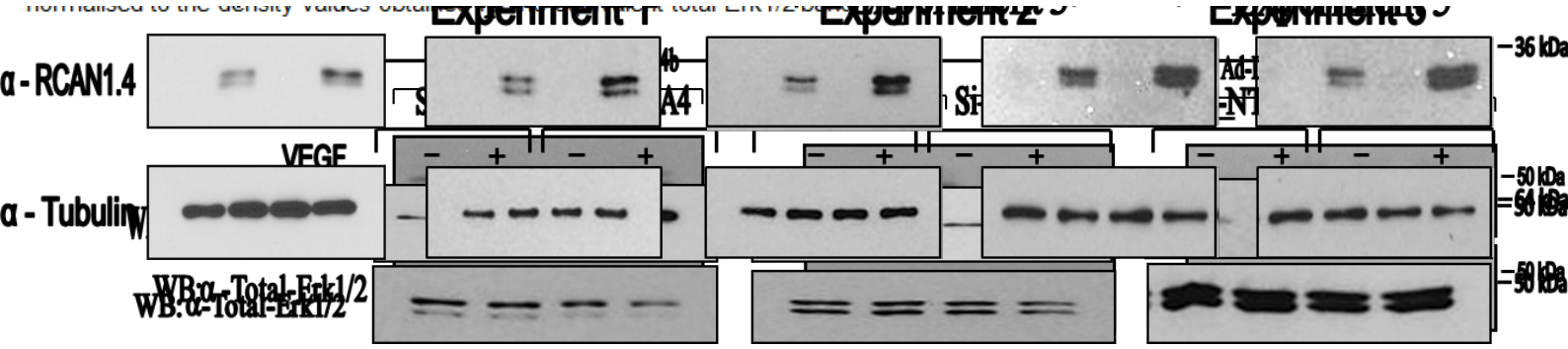
α-Tubulin
Si-PMCA4
36 kDa
64 kDa

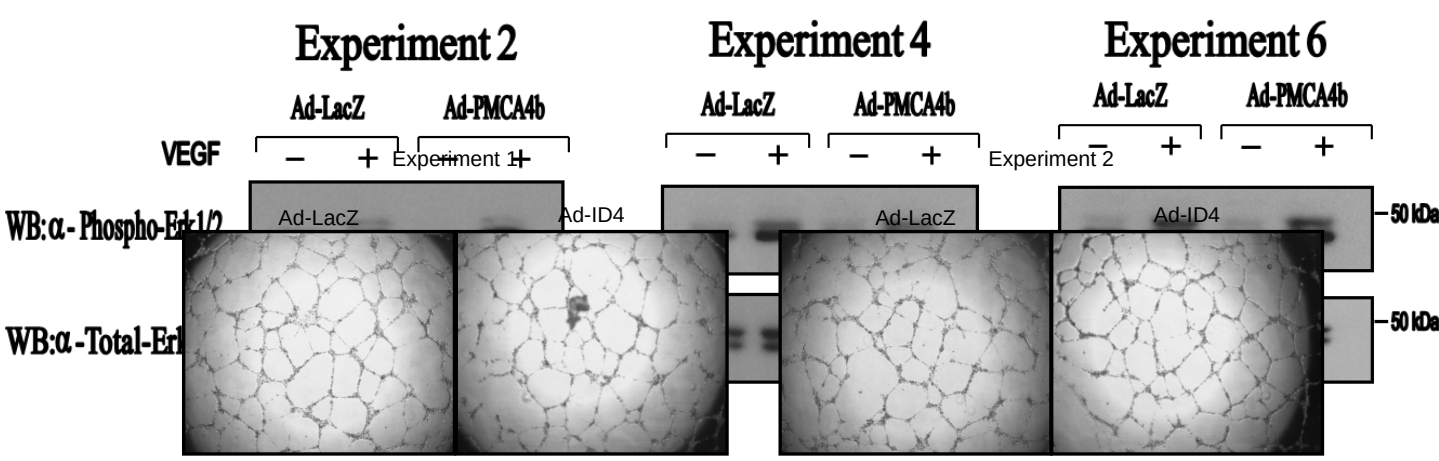

Experiment 4
Experiment 5
Experiment 6
2% FBS
2% FBS + VEGF
Si-NT
Si-PMCA4


Experiment 7
2% FBS
2% FBS + VEGF
Si-NT
Si-PMCA4

APPENDIX 11: Images used to quantify the amount of tube formation with knockdown of PMCA4. HUVEC were transfected with si-RNA corresponding to PMCA4 (si-PMCA4) or non-target (si-NT), plated onto matrigel and stimulated with 2% FBS or 2% FBS with VEGF (50 ng/ml). Images were taken after 24 hours incubation and the number of tubes counted. The value obtained for si-NT 2% FBS (control) was set as 100% and all other values calculated as a percentage relative to this value.

APPENDIX 12: Western blot images from two independent experiments, n=6 used to calculate the levels of phosphorylated (activated) Erk1/2 with ectopic expression of PMCA4. HUVEC transfected with Ad-LacZ or Ad-PMCA4b were unsitmulated (-) or stimulated with VEGF (25 ng/ml) (+) for 5 minutes. Western blots detecting phosphorylated Erk1/2 (WB:α-Phospho-Erk1/2) and total Erk1/2 (WB:α-Total-Erk1/2) were carried out. The density of bands was measured using Image J software and phosphorylation values for Erk1/2 were normalised to the total levels of Erk1/2 in each sample.





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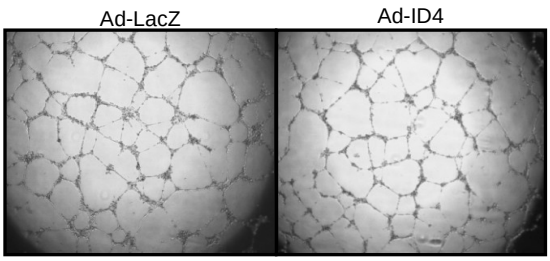
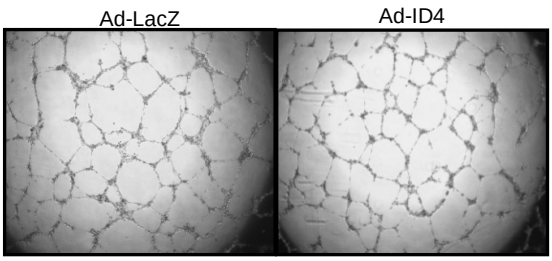


Experiment 4



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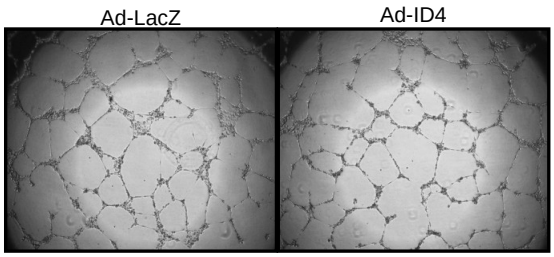

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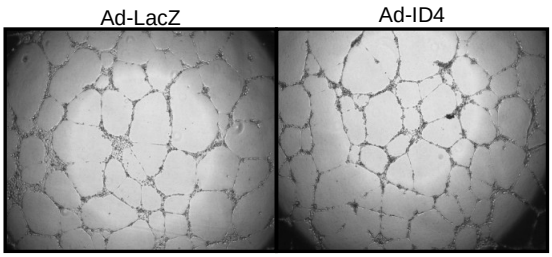
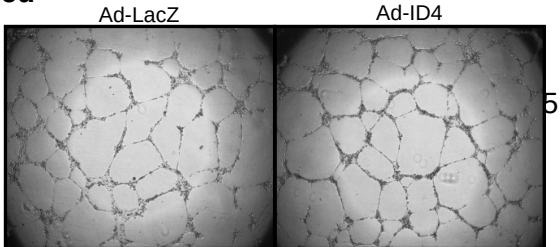
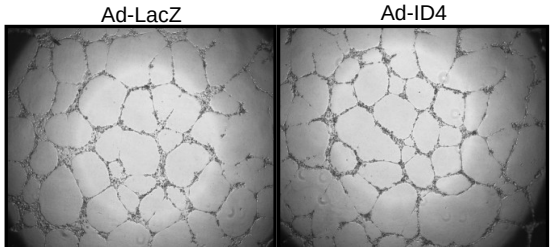


APPENDIX 15: Images used

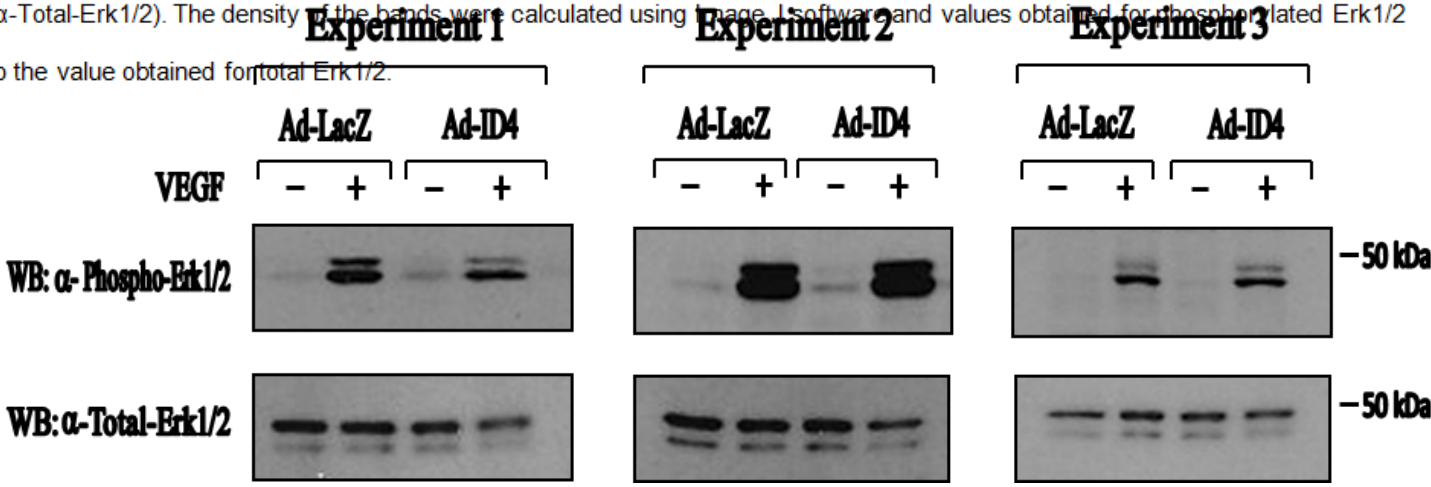
Experiment 7



Experiment 8



APPENDIX 16: Western blot images used to calculate the phosphorylation of Erk1/2 with ectopic expression of the interaction domain of PMCA4 (ID4). HUVEC were infected with Ad-LacZ (control) or Ad-ID4 and stimulated with VEGF (25 ng/ml) for 5 minutes (+) or left unstimulated (-). Western blot analysis was carried out using antibodies specific for phosphorylated Erk1/2 (WB:α-Phospho-Erk1/2) and total Erk1/2 (WB:α-Total-Erk1/2). The density of the bands were calculated using Image J software and values obtained for phosphorylated Erk1/2 normalised to the value obtained for total Erk1/2.



to quantify the amount of tube formation with over-expression of the interaction domain of PMCA4 (428-651) (ID4). HUVEC were infected with Ad-LacZ or Ad-ID4 and seeded onto matrigel in 2% serum. After 24 hours cells were fixed and images taken. The amount of tube formation was calculated by counting branching points and the value obtained for Ad-LacZ set as 100%. Other values were calculated as a percentage relative to this control.